Secretor status and immune response to *Neisseria* species

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Declaration

I declare that this thesis has been composed by myself and that the research reported therein has been conducted by myself or under my direct supervision.

Edinburgh, 20 June 1993

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I dedicate my work to my wife and to my parents

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Abstract

Non-secretors of ABO blood group antigens are over-represented among patients with meningococcal disease. The lower levels of both serum secretory antibodies reported, for some studies, for non-secretors have been suggested to compromise their defences against bacterial diseases. Conflicting results have been reported for differences in immunoglobulin levels between secretors and non-secretors; however, most of these studies measured total amounts of antibodies in serum and saliva by single radial immunodiffusion (SRID) assays and there have been no studies of their functional activities.

The objectives of the study were (1) to assess total and specific antibodies in sera and saliva of secretors and non-secretors with reference to carriage of meningococci and smoking; (2) to assess the ability of saliva from secretors and non-secretors to inhibit bacterial binding to epithelial cells; (3) to assess bactericidal activity of sera from secretors and non-secretors.

As a preliminary step, a sensitive precise and accurate enzyme linked immunosorbent assay (ELISA) was developed to assess the total and specific levels of IgA, IgM, IgG in serum and saliva. In a parallel trial, antibody levels obtained with the ELISA and SRID assay revealed that the ELISA was significantly more sensitive.

Total serum and salivary IgG, IgA and IgM and levels of these isotypes specific for *Neisseria lactamica* and five isolates of meningococci were determined by ELISA from material obtained from 357 pupils and staff of a secondary school in which an outbreak of meningitis occurred. There were no differences in total or specific levels of serum IgG, IgA or IgM or salivary IgG or IgA of secretors compared with non-secretors. Non-secretors had significantly lower levels of total salivary IgM ($p = 0.022$)and lower levels of IgM specific for *N. lactamica* and five meningococcal isolates. No correlation

between levels of serum and salivary IgM suggested that this IgM was produced locally and had not leaked from the serum. Although carriers had higher levels of antibodies than non-carriers, the effect of secretor status on antibody levels was still significant. Smoking had no effect on levels of total antibodies as those for neisseria.

A flow cytometric assay was developed to assess the effect of salivary antibodies on the attachment of meningococci to epithelial cells. Two types of saliva were tested, fresh saliva from individual donors and a pool from the school children. Both pooled and fresh saliva demonstrated significantly high levels of inhibitory activity. The inhibitory activities for pooled and fresh saliva obtained from secretors were statistically greater than that observed for non-secretors. The pool from which anti-meningococcal antibodies were absorbed retained a high level of inhibitory activity, suggesting factors in saliva other than antibodies inhibited the attachment of bacteria to epithelial cells. There was no statistical difference between the inhibitory activities of affinity purified IgA or IgM antibodies.

Most of the sera from secretors and non-secretors had high bactericidal titres against *N. meningitidis* NG:4:- isolate and *N. lactamica* . Bactericidal activity against capsulate strains was associated with high IgG levels to *N. lactamica.* Both carriers and secretors had significantly higher levels of IgG to *N. lactamica* in sera with bactericidal activity for each of the four capsulate strains compared with sera in which there was no bactericidal activity.

Abbreviation

BBBP	blood-brain barrier permeability
BEC	Buccal epithelial cells
BI	Binding index
BSA	Bovine serum albumin
CIE	Counterimmunoelectrophoresis
c.f.u	Colony forming unit
CNS	Central nervous system
CSF	Cerebrospinal fluid
CV	Coefficient variation
D.PBS+B	Dulbecco's phosphate-buffered saline
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein isothiocyanate
GC	Gonococcal culture
HRP	Horseradish peroxidase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
Le	Lewis
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
mIgA	monomeric IgA
MNYC	Modified New York City medium
OD	Optical density
OMP	Outer membrane protein

O/N	Overnight
PBS	Phosphate-buffered saline
PCB	phosphate citrate buffer
pIgA	polymeric IgA
PMNL	Polymorphonuclear lymphocyte
PRP	polyribosyl phosphate
RIA	Radioimmunoassay
RT	Room temperature
SC	Secretory component
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Se	Secretor
sIgA	Secretory IgA
sIgM	Secretory IgM
SRID	Single radial immunodiffusion
TNF	Tumour necrosis factor

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General Introduction

1.1 Meningococcal disease

Meningococcal disease, "epidemic cerebrospinal fever", is a world wide health problem, causing unpredictable epidemics with high mortality. The main clinical signs and symptoms were first described in 1805 by Vieusseaux (see Branham, 1940). Weichselaum (1887) (see Branham, 1940) who first recognized the causative bacterium in the cerebrospinal fluid (CSF) and called it *Diplococcus intracellularis meningitidis* due to its presence within leukocytes. Subsequent studies have established the meningococcus as the cause of epidemic cerebrospinal fever and places the organism in the genus *Neisseria* (Wollstein, 1907) (see Branham, 1940). The species *Neisseria meningitidis* is classified into different serogroups, serotypes and subtypes based on capsular and outer membrane protein antigens. Monoclonal antibodies to the lipooligosaccharide are also being used for strain differentiation.

Disease caused by *N. meningitidis* is limited to humans; there is no other natural host. When restricted to the oropharygeal mucosa, these infections are referred to as the "asymptomatic carrier state". Bacteraemic spread from the submucosal tissue is a rare event and is associated with much more serious conditions which can rapidly lead to death. The clinical spectrum of meningococcal disease ranges from mild, local infection to fatal septicaemia (Apicella, 1990).

1.2 Epidemiology of meningococcal disease

1.2.1 Geographical distribution

Meningococcal meningitis can be either endemic or epidemic. Epidemic meningitis is found in many countries. The largest epidemic occurred in Europe and in the United States during World War II. Major epidemics have continued in other parts of the

world. The serogroup A epidemic in Sao Paulo during 1974 had the highest incidence recorded, 370 cases per 100,000 (Peltola, 1983). Africa contains one of the worst affected areas known as the "meningitis belt" (Greenwood *et al.*, 1979). These epidemics occur every 8-12 years and last for 2-4 years. Most cases of meningitis within the African meningitis belt occur in the hot dry seasons of the year (Greenwood *et al.*, 1979).

In England, the United States and Norway, peak incidences have been recorded during the winter season (Fraser *et al.*, 1972; Bøvre and Gedde-Dahl, 1980; Band *et al.*, 1983). The bacteriologic, environmental and host-specific factors that result in the epidemics have been studied but remain largely unknown.

1.2.2 Age

The most striking epidemiologic feature of bacterial meningitis is the age distribution of cases. Meningococcal disease can occur at any age in previously healthy individuals; however, the disease is seen most frequent in young children between 6 months and 4.5 years. These are infants and young children in whom protective maternal antibody has waned and who are not yet able to produce an effective long-term response to polysaccharide antigens (Greenwood, 1984). The incidence rises again in teenagers (Cartwright *et al.*, 1986). A low level of disease is seen in adults between 15 and 44 years before it increases again slightly in older adults and in the elderly. The age distribution can vary depending on the bacterial strain and the population affected.

In the United Kingdom at least one child in every 1000 develops acute bacterial meningitis by the age of 10 (Greenwood, 1984). In some areas of Africa, acute bacterial meningitis in childhood can be as high as 1 in 100 (Greenwood, 1984).

1.2.3 Carrier state and transmission

Direct comparison between studies must be made with caution since many techniques and identification schemes have been used in meningococcal carriage studies. *N. meningitidis* is carried asymptomatically in the nasopharynx by 5 - 20% of the adult population in non-epidemic periods (Greenfield *et al.*, 1971). Although meningococcal disease peaks during winter months, no significant difference in carriage was demonstrated among Greek recruits examined in summer months compared with winter months (Kremastinou *et al.*, in press a). Higher rates of meningococcal carriage have been demonstrated in household contacts, hospital personnel and children attending day-care centres (De Wals *et al.*, 1981; Broome, 1986). There seems to be controversy about the carrier prevalence related to sex. In the Stonehouse survey and in a study in the Faroe Islands, there was a higher prevalence of carriage of *N. meningitidis* among males (Cartwright *et al.*, 1987; Olsen *et al.*, 1991); others have found no difference in *N. meningitidis* carriage between the sexes (Gold *et al.*, 1978a; Blackwell *et al.*, 1990; Blakeborough *et al.*, 1982; Kremastinou *et al.*, in press b). A higher carriage rate found for the 15 - 19 age range (Cartwright *et al.*, 1987; Blackwell *et al.*, 1992a).

Wide variations in duration and acquisition of carriage have been demonstrated (Greenfield *et al.*, 1971; Blakeborough *et al.*, 1982). Efficient transmission of meningococci is associated with close contacts, crowding, and poverty (Cartwright *et al.*, 1987; De Wals *et al.*, 1983) and might be influenced by co-existent viral infection (Wall, 1988). Such environmental and sociologic factors might lead to the higher carrier rates seen in house-hold contacts of cases (Kaiser *et al.*, 1974), carriers (Marks *et al.*, 1979) or closed populations such as military recruits and boarding school pupils. In studies of Greek military recruits, the carriage rate was 25% during the summer when 4.9% had symptoms of upper respiratory infection and only 27% in the winter when 46.4% had symptoms of viral infection (Kremastinou *et al.*, in press a).

1.3 Classification of *N. meningitidis*

N. meningitidis are encapsulated Gram negative diplococci with a flattened shape and an average size of 0.6 x 0.8 μm. Much effort has been spent on their classification.

1.3.1 Serogroups

There are 13 different serogroups of *N. meningitidis* based on the antigenic specificity of the capsular polysaccharide: A, B, C, D, X, Y, Z, 29E, W135, H, I, K, and L (see Apicella, 1991). Approximately 90% of meningococcal disease world-wide is caused by serogroups A, B or C (reviewed by Schwartz *et al.*, 1989). Historically, strains of serogroups A have been the cause of major epidemics and pandemics. Most African epidemics have been caused by meningococci belonging to serogroup A. The reasons for the association between serogroup A and epidemics are unclear. Strains of serogroup C have less frequently been responsible for major epidemics (Peltola, 1983, Olyhoek *et al.*, 1985). Serogroup B meningococci have generally been associated only with sporadic cases and localized outbreaks of disease; but, beginning in the 1970's, epidemics caused by serogroup B strains have occurred in several European countries, Norway, the United Kingdom and Iceland (Peltola, 1983; Poolman *et al.*, 1985). There have been outbreaks due to this serogroup in Chile and Cuba (Cruz *et al.*, 1990). Serogroup Y and W135 account for almost all the rest (Ashton *et al.*, 1983). Strains of other serogroups as well as non-groupable strains are usually non-pathogenic carrier isolates (Craven *et al.*, 1980).

Only capsulate meningococci are pathogenic, and non-capsular mutants of virulent strains lose their virulence for mice (Bøvre *et al.*, 1983). Systemic isolates have more capsule than pharyngeal strains. Non-capsular strains which account for 20-50% of the nasopharyngeal isolates from healthy carriers usually do not cause systemic meningococcal disease (Craven *et al.*, 1980). This might be explained by the inverse relationship between the amount of capsular material and both the adherence to

epithelial cells and the antiphagocytic properties of the polysaccharide capsule (Craven *et al.*, 1980). Protective immunity to serogroups A and C meningococci is induced by the purified polysaccharide A and C vaccines (Griffiss, 1982; Griffiss *et al.*, 1987a). In contrast, serogroup B strains seem not to induce bactericidal anti-polysaccharide antibodies in man (Wyle *et al.*, 1972; Zollinger and Mandrell, 1983).

1.3.2 Outer membrane proteins (OMPs)

Serogrouping by the capsular polysaccharide is of limited value in epidemiological research because one serogroup often predominates at any one period or in a geographical region (Peltola, 1983). Meningococci have been subdivided into a series of serotypes and subtypes based upon immunologically distinct OMPs.

Five classes of OMPs can be demonstrated by chromatography (Frasch and Gotschlich, 1974; Mocca and Frasch, 1982), but the functions of the majority of these are not clearly understood. The class 1 and class 2/3 proteins are not subject to antigenic shift but show variations between strains and are used to determine subtype and serotype specificity, respectively. The class 5 proteins elicit bactericidal antibodies during infection (Poolman *et al.*, 1983); they might play role in the adhesion process (see Verheul *et al.*, 1993) but undergo antigenic shift and by this means they can escape recognition by the immune host response (Tinsley and Heckels, 1986). In contrast, the class 4 protein appear to be highly conserved between meningococcal strains and shows considerable homology with the equivalent protein (PIII) present in gonococci (Klugman *et al.*, 1989). Evidence exists that antibody to this protein blocks the bactericidal activity of antibodies directed against other OMPs (Munkley *et al.*, 1991). Table 1.1 shows the OMPs (porins) designated for *N. gonorrhoeae* and its counterpart for *N. meningitidis* (adapted from Hitchcock, 1989).

Table 1.1: OMPs of *N. gonorrhoeae* and *N. meningitidis*

N. gonorrhoeae	*N. meningitidis*
Protein I (PI)	Class 1 Class 2 and 3
Protein III (PIII)	Class 4
Protein II (PII)	Class 5

Adapted from Hitchcock, 1989

The OMP serotyping is based upon antigenic differences of the most stable proteins of classes 2 and 3; and at least 20 to 30 serotypes have been described (Apicella, 1991). In addition, subtyping to distinguish between strains based on class 1 OMPs, and, to a lesser extent, upon differences among class 5 OMPs have been developed. Serotyping based on OMP antigens offers a more detailed approach to the epidemiology of meningococcal disease and has been a valuable tool in these studies (Frasch, 1977). Significant numbers of isolates are, however, non-typable. The endemic form of the disease appears to be caused by a heterogeneous collection of serotypes whilst a single serotype is usually involved in epidemics (Apicella, 1990). There are difficulties with serogroup A meningococci because certain serotype proteins are apparently invariant (Frasch *et al.*, 1985). Whole-cell ELISA is used for sero- and subtyping of *N. meningitidis* (Abdillahi and Poolman, 1987). This technique could discriminate clearly between strains carrying the antigens by use of monoclonal antibodies.

The OMP serotype 2 had been associated with disease-causing isolates of the serogroup B meningococcus during epidemics in the United States and Western Europe during the 1970s. This has been replaced by epidemics of organisms expressing other OMP serotypes (4 and 15). In Europe, at first in Norway, but later in other European countries (Poolman *et al.*, 1986), the phenotype associated with epidemics and prolonged outbreaks is B:15:P1.7,16. In England and Wales, this phenotype has been responsible for meningococcal disease since 1980. Epidemiologically this has been characterized by the persistence of infection in certain communities over a period of several years (Cartwright *et al.*, 1986).

Kasper and colleagues (1973b) found that bactericidal activity in convalescent phase sera from adults correlates better with titres of antibodies to serotype antigens than with titres to the serogroup B polysaccharide. The serotype and serosubtype proteins are being explored as vaccine candidates for meningococcal disease.

1.3.3 Lipooligosaccharide

Lipooligosaccharide (LOSs) are surface glycolipids which are a major component of the outer membranes of *N. meningitidis*. LOS can be utilized for classification into LOS serotypes (Frasch *et al.*, 1985). At present, there are 13 different lipooligosaccharide immunotypes (L1-L13) (Achtman *et al.*, 1992) and monoclonal antibodies against these epitopes have been produced. The serogroup B and C LOS antigens (L1-L8) are shared, whereas the LOS antigens of the serogroup A (L9-L12) appear to be unique to that serogroup (Achtman *et al.*, 1992). L2 - L3 were found to be most prevalent among serogroup B and C meningococci (Achtman *et al.*, 1992). The scheme seems to be prone to errors, especially for serogroup A it is not possible to discriminate between L11 and L12 (Poolman, personal communication). The distribution of LOS immunotypes among serogroup B meningococci might become of special interest, because the LOS is a potential vaccine candidate (Poolman, 1990).

1.3.4 Enzymatic typing

The clonal typing method has now been used in epidemiological studies of serogroup A (Olyhoek *et al.*, 1987) and of other serogroups (Caugant *et al.*, 1987). The method is based on either the isoenzyme analysis by electrophoretic mobilities of soluble cytoplasmic enzymes (Olyhoek, 1985, Selander *et al.*, 1987) or on a combination of isoenzyme analyses and OMP analysis (Achtman and Pluschke, 1986). Clonal analysis has not been found to correlate with serotyping results for serogroup B and C strains (Caugant *et al.*, 1987); but, serotyping results with serogroup A strains showed a clear correlation with clonal analysis (Crowe *et al.*, 1989).

These methods were capable of distinguishing between strains of the same serogroup and have provided additional insight into the epidemiology of meningococcal disease. This method provides a high resolution system for the characterization of all isolates of

N. meningitidis and provides estimates of the extent of variation in the chromosome as a whole and of the genetic relatedness of the strains (Selander *et al.*, 1987).

1.3.5 Molecular methods

New molecular techniques such as the restriction fragment length polymorphism (RFLP) typing method have been developed for *N. meningitidis*. The RFLP method is based upon detection of minor differences in base sequence which often can not be discerned in the phenotype (Tompkins *et al.*, 1986). Fox and colleagues (1991) found that the RFLP technique was highly discriminatory and reproducible; and RFLP data were consistent with those from the alternative typing methods such as the isoenzyme electrophoretic typing method suggesting that RFLP might be useful as an additional tool for epidemiological typing of meningococci.

1.4 Pathogenesis of meningococcal disease

A microorganism must successfully take many sequential steps to cause disease. This section will focus on the ultrastructural events and possible molecular mechanisms of the steps leading to disease due to *N. meningitidis*. The sequence of events leading to disease is generally held to be:

1) colonization of the oro- or nasopharynx (carrier state).

2) a meningococcal bacteraemic phase in which the bacteria enter the blood stream and give rise to meningococcaemia;

3) involvement of the inflammatory response in damage to skin, eyes, joints, adrenal glands or meninges with various clinical symptoms.

1.4.1 Attachment and transgression of the mucosal epithelium

Only a few individuals colonized with meningococci in their upper respiratory tract develop meningococcal disease. This might, in part, be due to differences between the meningococcal strains in their pili, capsular polysaccharides, OMP, IgA protease and LPS composition which appear to be involved in the bacterial attachment to the mucosa or invasiveness of the meningococci (De Voe, 1982; Poolman *et al.*, 1983).

Early investigations of meningococcal pathogenesis implicated the nasopharynx as the natural habitat of the meningococcus. This site acts as a focus from which the organism can be transmitted to other individuals and as the initial site of mucosal colonization preceding systemic disease. Although the nasopharynx is important in the maintenance of the organism in the environment, the factors that determine attachment of meningococci predominantly to this site rather than to other mucosal surfaces are poorly understood.

After deposition onto the mucus, the bacteria must adhere rapidly to the underlying cell surface if they are not to be removed by the muco-ciliary escalator. Colonization probably depends largely on ligands or adhesins on the organisms interacting with receptors on host cells. Attachment of microorganisms might vary with different cell types and with cells of the same type under different conditions (Svanborg-Eden *et al.*, 1980).

Pili or fimbriae, long hair-like structures, are important bacterial adhesins. Adherence of *Escherichia coli* by means of fimbrial antigens to epithelial cell receptors in the human urinary tract is an important event in the pathogenesis of acute pyelonephritis (Lomberg *et al.*, 1983). Uropathogenic *E. coli* strains attach to glycolipids of the globo-series on urogenital epithelial cells, antigens of the P blood group system. Information about bacterial ligands responsible for attachment to host cell receptors has been successfully applied in the development of vaccines that prevent disease by

inducing attachment blocking antibodies. Jones and Rutter (1972) observed protection of newborn piglets against *E. coli* diarrhoea after immunization of the pregnant sows with purified K88 fimbriae.

Pili are found on 80% of primary meningococcal isolates from nasopharyngeal of carriers as well as from cerebrospinal fluid of meningitis patients (De Voe and Gilchrist, 1975). Attachment of pilate meningococci differed markedly among epithelial cells from different sites. There was significantly more attachment of meningococci to nasopharyngeal and buccal epithelial cell compared with cells from the urethra, transitional bladder or anterior nasal epithelial cells (Stephens and McGee, 1981). Pili have been identified on *N. meningitidis* and are associated with enhanced attachment to the host cells and increased virulence (Silverblatt and Cohen, 1979). On the other hand non-piliate strains more easily escape from phagocytosis by PMNLs (Buchanan *et al.*, 1978), and systemic meningococcal isolates are less piliate than pharyngeal strains (Craven *et al.*, 1980). Pili were demonstrated on the bacteria isolated from CSF from a patient with meningococcal meningitis indicating a possible role for pili in the later stages of disease (Stephens *et al.*, 1982). The protective role of anti-pili antibodies remains uncertain (De Voe, 1982).

Capsular polysaccharide, outer membrane proteins and lipooligosaccharide might also influence the attachment of *N. meningitidis* to human cells (Craven and Frasch, 1978). The presence of the capsule has been suggested to decrease the adherence of meningococci to mucosa and human erythrocytes (Craven *et al.*, 1980; Stephens *et al.*, 1993). The role of these bacterial components on binding of the organism needs to be assessed with reference to the effects of the host's mucosal defences: secretory immunoglobulins; mucus; ciliary action and phagocytes (McNabb and Tomasi, 1981).

Mechanisms that affect some local defences (e.g. damage to ciliary activity or IgA protease) might have evolved in meningococci. Damage to ciliary function is a

characteristic of pathogenic bacteria that infect mucosal surfaces. *Bordetella pertussis* causes damage to ciliated cells after direct attachment, and *N. gonorrhoeae* damages ciliated cells from a distance by release of soluble substance (e.g. LPS) (Stephens *et al.*, 1987). *N. meningitidis* also exerts a cytoxic effect on ciliated human nasopharyngeal mucosal cells by means of soluble substances (Stephens and Farley, 1991). In addition, concurrent upper respiratory tract viral or mycoplasma infections have been postulated to enhance invasion by meningococci at the nasopharynx (Schwartz *et al.*, 1989), possibly by disrupting the mucociliary barrier or epithelium.

Stephens and colleagues (1983b) found that meningococci appeared to adhere solely to the microvilli of non-ciliated cells of the epithelium in human nasopharyngeal mucosa. The microvilli then become restructured and surround the microorganisms. Simultaneously with this attachment phase (up to 12 hours post-infection), a rapid decrease in the ciliary activity together with sloughing of the ciliated epithelial cells was observed. The meningococci attached to the non-ciliate cells were then engulfed within phagocytic vesicles. Despite the apparent absence of the endocytic vacuoles in the basal aspects of the cells, meningococci appear in the subepithelial tissues 18 - 24 hours post-infection. Unlike gonococci invading human fallopian tube mucosa (McGee *et al.*, 1988), meningococci were not observed penetrating the apical junctions and intercellular spaces.

1.4.2 Bacteraemia

Once the mucosal barrier is crossed, bacteria might gain access to the blood stream and must overcome additional host defence mechanisms. Surface encapsulation is the most important bacterial factor which effectively inhibits neutrophil phagocytosis. It also has a role in resistance to bactericidal activity mediated by the alternative complement mediated pathway. This enhances blood stream survival and facilitates intravascular replication to high bacterial densities.

The terminal complement components (C5-C9) are especially important in host defences against meningococcal infections and congenital or acquired deficiencies in any of these components predispose individuals to recurrent or chronic infections with *N. meningitidis* (Ross and Densen, 1984; Rosen *et al.*, 1988). The bacteraemia associated with meningitis is usually assumed to be primary; that is, it follows nasopharyngeal colonization and leads to central nervous system (CNS) invasion.

1.4.3 Inflammation and damage in CNS

LOS of meningococci is probably the most potent bacterial endotoxin known. The effect of endotoxins released from the bacteria on the host system can be due to direct action on the tissue (reviewed by Jacobs and Tabor, 1990). Systemic isolates of meningococci release higher amounts of LOS than carrier strains (Andersen *et al.*, 1987). LOS of meningococci induce a complex system of reactions which might lead to septic shock, disseminated intravascular coagulation and multiple organ failure (De Voe 1982). As a consequence, LOSs appears to play a major role in modulating the course of the disease and determining the outcome of the disease (Mœland, 1980).

LOS activates the complement system (Greenwood *et al.*, 1976) and stimulates the release from leukocytes of a number of physiologically active cytokines (Rietschel *et al.*, 1982), including tumor necrosis factor (TNF) (Waage *et al.*, 1986). Waage and colleagues (1989a) measured the level of TNF in patients with meningococcal meningitis. The level was high in 10 of 11 patients who died compared with 8 of 68 survivors from the disease. Interleukin 1 (IL-1) and interleukin (IL-6) are present in the systemic circulation during the development of septic shock (Waage *et al.*, 1989b). These cytokines are also produced in the CSF. Interleukin (IL-8) appears to participate in the complex cytokine network during the initial phase of systemic meningococcal infections (Halstensen *et al.*, 1993).

Damage to the host results from the inflammatory changes causing injury to the vascular endoththelium, which leads to increased blood-brain barrier permeability (BBBP) and activation of the coagulation cascade. Depending on the potency and duration of the inflammatory stimuli, the BBBP is altered to different degrees, and serum proteins and other macromolecules penetrate into CSF. The consequences include severe brain oedema, increased intracranial pressure and reduced cerebral blood flow. The interaction of all these events will eventually lead to neuronal injury and to the irreversible focal or diffuse brain damage that can occur as consequence of bacterial meningitis.

1.5 Immune responses to meningococci

Once a microorganism has penetrated subepithelial tissues and invaded the circulatory system, a number of immune mechanisms defend the host against proliferation of the bacteria and establishment of the disease state. The process of eliminating invading encapsulated bacteria from the circulatory system is based on three factors: phagocytosis; production of humoral antibodies; and the activation of complement.

1.5.1 Phagocytosis in the non-immune host

Normal human serum has bactericidal and opsonising activities against *N. meningitidis*. Most of the attention has been given to the serum bactericidal activity against meningococcal disease (Craven *et al.*, 1982). Less work has focused upon the killing of the bacteria by phagocytes (Ross *et al.*, 1987); therefore, little is, known about the relative importance of phagocyte-mediated killing of meningococci compared to humoral mediated bactericidal activity.

There is increasing evidence for different mechanisms of protection against various serogroups. Complement-dependent phagocytosis is relatively more important in defence against serogroup B meningococci than against serogroups A, C, Y and W135

(Ross *et al.*, 1987). This has recently been supported by the report that monoclonal antibodies against the serogroup B polysaccharide are opsonic but not bactericidal in the presence of human complement and are highly protective in three animal models (Raff *et al.*, 1988). The serogroup B meningococcal sialic acid capsules appear to inhibit activation of the late components of the alternative complement pathway. This compromises the bactericidal defence against serogroup B meningococcal disease in the nonimmune host, especially in individuals with deficiency in early complement component (Ross *et al.*, 1987; Jarvis and Vedros, 1987).

1.5.2 Production of antibodies and their function

1.5.2.1 IgM and IgG antibodies

Low bactericidal activity is a major factor in the development of systemic infections with *N. meningitidis* (Goldschneider *et al.*, 1969a; Griffiss and Bertram, 1977; Skevakis *et al.*, 1984). Families and individuals with IgM deficiency are at increased risk of disseminated meningococcal disease (Hobbs *et al.*, 1967; Jones *et al.*, 1973). There are wide variations in the efficiency with which different antibody classes can function in complement killing. Griffiss and Bertram (1977) reported that natural antibodies to meningococci are predominantly IgM. They emphasized the superiority of IgM antibodies over IgG as an activator of the classical pathway in the bactericidal reaction; there are large differences in the relative activities of the two antibody classes. Together they probably account for the rapid classical pathway-mediated bacterial killing in the unchelated assay system (Skevakis *et al.*, 1984).

1.5.2.2 IgA antibodies

Early studies with purified IgA suggested that this antibody could activate bacteriolysis in the presence of complement and lysozyme (Adinolfi *et al.*, 1966). Subsequent studies have failed to confirm this finding (Eddie *et al.*, 1971; Heddle *et al.*, 1975);

and, it has been suggested that the IgA fraction examined by Adinolfi and colleagues might have been contaminated with small amounts of immunoglobulins of other classes (Eddie *et al.*, 1971; Rowley, 1973). The finding that circulating, strain specific IgA antibody to *N. meningitidis* isolated from convalescent phase sera is capable of blocking the complement mediated bacteriolytic activity of IgG and IgM separated from the same sera (Griffiss *et al.*, 1975) raised the possibility that the blocking by IgA might be responsible for the absence of the lytic activity in the sera of some susceptible individuals. This blocking effect is antigen-specific, *i.e.*, both the blocking IgA and the lytic antibody compete for the same antigenic site (Griffiss *et al.*, 1975). Later reports have, however, provided evidence that IgA can activate the alternative complement pathway in bactericidal reactions against *E. coli* (Sirotak *et al.*, 1976). Chemically aggregated human myeloma IgA1 and IgA2 as well as Fc fragments can function as classical pathway activators (Burritt *et al.*, 1977). In addition, Hiemstra and colleagues (1988) demonstrated that IgA1 and IgA2, in both monomeric and aggregated forms, activate complement via the alternative pathway.

This controversy over the ability of IgA to activate complement has not been settled. Griffiss (1982) presented an interesting model of the epidemic behaviour of meningitis due to *N. meningitidis*. The experimental data for this model is based on the observation that the acute sera of military recruits with meningococcal meningitis contains IgA antibodies which block the bactericidal effect of IgM and IgG antibodies against the pathogen (Griffiss and Bertram, 1977). It was suggested that IgA 'blocking' antibody arises from coincidental enteric exposure to non-pathogenic enteric organisms which express structurally similar surface antigenic determinants. This results in the stimulation of cross-reactive blocking antibodies which render the host susceptible if exposed to the meningococcus. This hypothesis might explain the epidemic spread of the meningococcus through populations.

In contrast to this earlier work, Jarvis and Griffiss (1989) reported that IgA1 can initiate complement mediated lysis of serogroup C meningococci through the classical pathway when bound to specific outer membrane proteins. The initiation of lysis is an Fc-dependent process; however IgA1, can also function as a blocking antibody when bound to the meningococcal polysaccharide capsule. Recently Jarvis and Griffiss (1991) found that human IgA1 and its Fab and $F(ab')_2$ fragments can inhibit IgG-initiated lysis of serogroup C meningococci via either the classical or alternative pathway before and after the organism is exposed to IgG. The blockage is not dependent on the Fc portion binding to capsular polysaccharide epitopes. They suggested that the biologic effect of IgA1 is dependent primarily upon the antigen to which IgA1 binds rather than on the structure of the IgA itself.

1.5.2.3 Development of natural antibodies to meningococci

Exposure of many Gram-negative bacteria to suitable concentrations of human or animal serum results in loss of viability and sometimes dissolution of the bacterial cells. An inverse relationship exists between the development of bactericidal immunity and risk of meningococcal disease (Goldschneider *et al.*, 1969b).

In serological studies on healthy human populations, the presence of antibodies having a specificity for the capsular polysaccharides of serogroups A, B, and C meningococci is frequently detected, and this cannot be satisfactorily explained by asymptomatic carriage (Robbins, 1979). These antibodies probably result from exposure to structurally similar antigens among the nonpathogenic bacteria found on the mucosal surface.

Neonates have a low incidence of meningococcal disease, probably due to anti-meningococcal IgG obtained from the mother by placental transfer. The development of bactericidal antibodies reactive with serogroupable meningococci confers the increased resistance observed in later childhood. These antibodies are thought to

develop as a consequence of antigenic stimulation by carriage of *N. meningitidis* (especially non-serogroupable strains), colonization with the genetically closely related but rarely pathogenic *N. lactamica* (Reller *et al.*, 1973; Gold *et al.*, 1978a) or colonization with commensal enteric bacteria which express structures antigenically similar to the capsular and somatic antigens of meningococci (Robbins *et al.*, 1974). Gold and colleagues (1978a) found that 40% of children who carried *N. lactamica* developed increased titres of bactericidal antibody reactive with meningococci of serogroups A, B and C.

Antibodies against the capsular polysaccharide of serogroups A and C meningococci are also acquired in early childhood, although at markedly different rates. Most infants have detectable anti-A antibody by the age of 1 year but do not have detectable anti-C antibody until 2-3 years of age (Gold and Lepow, 1976). These anti-polysaccharide antibodies are acquired in the absence of detectable carriage of serogroup A or C meningococci (Gold *et al.*, 1978b).

1.5.2.4 Antibody responses to infection

After infection, a strong immune response to the capsular antigen occurs: IgM, IgG and IgA to these polysaccharides can be measured. These are of the same order of magnitude as those seen in postvaccine sera (Kayhty *et al.*, 1981).

The antigens inducing the antibody following disease include the cell membrane proteins and lipooligosaccharide as well as serogroup specific capsular polysaccharides. Frasch (1977) has shown that children under 2 years of age can make antibody to the protein serotype expressing the 2 antigen after recovery from disease due to serogroup B or C serotype 2 meningococci of antigens. Adult levels of antibody to serotype 2 are not reached until age 10. Zollinger and colleagues (1974) demonstrated that, although the titres of antibody to the protein and lipooligosaccharide antigens are the same after disease caused by serogroups B or C,

very little antibody is induced by the serogroup B polysaccharide in comparison to the C polysaccharide. Most of the antibody resulting from infection with serogroup B meningococci was directed against the cell membrane protein with little antibody found against the lipooligosaccharide or capsular polysaccharide (Zollinger *et al.*, 1974).

Natural infections with serogroup B meningococci do not appear to result in a strong antibody responses to the serogroup B polysaccharide (Brandt *et al.*, 1972; Zollinger *et al.*, 1974). In addition, Kasper and colleagues (1973a) found that antibodies to serogroup B polysaccharide were apparently not bactericidal for all serogroup B strains.

1.5.3 Bactericidal assay

The specificity of human antibodies which are bactericidal for meningococcal serogroup B has been examined by a number of investigators, often with conflicting results (Goldschneider *et al.*, 1969b; Kasper *et al.*, 1973a; Reller *et al.*, 1973; Frasch, 1979; Craven *et al.*, 1982). Some of the discrepancies might be attributable to differences in complement sources and to differences in the assays used. The source of complement and the bacterial test strain are critical (Griffiss *et al.*, 1987b). The majority of investigators of bactericidal phenomena have utilized serum from humans (Olling, 1977); others have used domestic (Mittal and Ingram, 1975) or laboratory animals (Collins, 1967) as a source of complement. Zollinger and Mandrell (1983) have demonstrated that human antibodies to meningococcal serogroup B polysaccharide are strongly bactericidal with rabbit complement but not with human complement; and, antibodies that were bactericidal with human complement were primarily directed against non-capsular antigens.

No single technique has been recognized as a readily acceptable and widely applicable standard assay for estimation of serum bactericidal activity. Many variations exist in both bactericidal assay techniques and analysis of results. Investigation of serum

bactericidal activity involves exposure of a suspension of viable organisms to a suitable concentration of antibody and complement, incubation at the optimum temperature for complement activity and determination (after suitable periods of time) of the absolute concentration of viable organisms by some form of counting. The differences among the data obtained by different investigators might be a reflection of the source of complement, antibody isotype, antibody avidity, the presence of blocking antibody and the resistance or sensitivity of the organism to complement mediated lysis.

1.5.4 The role of complement

It is now clear that activation of complement by Gram-negative bacteria can occur via the classical or the alternative pathway. Killing of Gram-negative bacteria via the classical pathway normally requires all nine complement components. There are bacteria that appear to be resistant to serum bactericidal systems; these resistant strains are frequently isolated from bacteraemic patients, while the majority of related serum-sensitive strains are isolated from mucosal surfaces of normal individuals or patients without invasive disease. Both serum sensitive and serum resistant strains of *N. gonorrhoeae* activate complement and bind the complement components to their surfaces; however, the elements on the bacterial surface to which complement is bound differ for the serum sensitive and serum resistant forms (Joiner *et al.*, 1985). Antibodies directed against outer membrane components bind, activate complement and kill encapsulated strains of meningococci (Goldschneider *et al.*, 1969b; Frasch, 1979). Killing of the meningococcus and the gonococcus appears to require the late components of complement and the lytic attack mechanism (Ross and Densen, 1984). Serum bactericidal reactions might be of protective importance *in vivo* because individuals with defects of the membrane-attack complex sequence of complement (C5-C9) frequently develop systemic infections with neisseriae, despite the presence of normal antibody levels and intact C3 opsonic functions (Ross and Densen, 1984). The occurrence of repeated meningococcal infections in individuals with deficiencies in the

late complement components provides further evidence of the importance of the lytic system in meningococcal immunity.

Inherited complement deficiency states have been found in association with 10%-30% of the sporadic adult cases of meningococcal disease (Ross and Densen, 1984). An intact classical complement pathway might be important in resisting infection with *N. meningitidis* especially in families with properdin deficiency (Sjoholm and Nilsson, 1985). Individuals with inherited deficiency of properdin have a functional classical pathway of complement, and vaccination might be protective (Soderstrom *et al.*, 1989). Complement-dependent phagocytosis requires complement activation only through C3.

1.6 Mucosal immunity

The molecular basis for local immunity was established when Tomasi and colleagues (1965) confirmed that external secretions contained a unique immunoglobulin called secretory IgA (sIgA). Subsequent studies showed that sIgA was locally produced (Tomasi and Bienenstock, 1968; Ogra and Karzon, 1969); its titre in secretions correlated better than serum levels with protection against infectious disease (Perkins *et al.*, 1969). Local application of antigen was the most efficient method of stimulating production of this isotype (Waldman and Bergmann, 1984). Immunity at mucosal surfaces of children and adults is brought about mainly by sIgA and sIgM which represent quantitatively the most important immune responses at these sites (Brandtzaeg, 1989). In general, natural infections acquired via the respiratory or intestinal mucosa appear to be the most effective means of inducing specific antibody responses at these portals of entry as well as at other distant mucosal sites.

1.6.1 Production of antibodies

For effective transport of IgA into external secretions, the most important prerequisite is its polymeric configuration. Although initially sIgA was thought to be assembled from

monomeric IgA (mIgA) by complexing with secretory component (SC) after its secretion from cells (South, *et al.*, 1966), subsequent studies showed that polymers are formed within IgA-producing cells before their selective transport into external secretions (Lawton and Mage, 1969; Bienenstock and Strauss, 1970). Brandtzaeg (1974) proposed a model of IgA transport in which SC functioned as a surface receptor for polymeric J-chain-containing IgA or IgM on various epithelial cells, and evidence for this concept was obtained in different studies (Brandtzaeg, 1978; Nagura *et al.*, 1979). SC is the epithelial cell receptor for polymeric immunoglobulins responsible for their selective transport (Brandtzaeg, 1985; Vaerman, 1987). After formation of SC in the rough endoplasmic reticulum of epithelial cells and glycosylation in the Golgi complex, it is transported to the laterobasal membrane where it acts as a receptor for polymeric IgA (pIgA) or IgM. SC-immunoglobulin complexes are translocated by endocytosis to the lumen. This translocation starts with a non-covalent interaction between the polymeric immunoglobulin and the membrane bound SC. The complex is endocytosed in vesicles and transported to the other pole of the cell. The translocation ends after fusion of the vesicle with the cell membrane and splitting of the extracytoplasmic SC. SC is produced by epithelial cells irrespective of the presence of pIgA or IgM, and it is found in many secretions in free as well as in IgA- or IgM-bound forms. The J chain is responsible for the selectivity of the transport because it induces a particular conformation in polymeric immunoglobulins required for strong SC binding (Vaerman, 1987). These observations of a specialized molecule in secretions provided an explanation for specific immunological resistance to mucosal infection existing in the absence of demonstrable serum antibody.

1.6.2 Secretory IgA

In humans there are two subclasses of IgA, IgA1 and IgA2 which are the products of separate immunoglobulin heavy chain genes. In secretions, the two subclasses are present in approximately equal concentration (Delacroix *et al.*, 1982), but they are not

equally distributed between serum and the mucosal immune system. IgA1 is markedly enriched in serum and constitutes 85% of serum IgA (Delacroix *et al.*, 1982, 1983). IgA2 exists as two allotypic variants IgA2m[1] and IgA2m[2]. Although 95% of the IgA in secretions is polymeric, only 12% of the IgA in serum is in this form, 88% being monomeric (Delacroix *et al.*, 1983).

Although IgA is synthesized locally and secreted throughout the mucosal surfaces of the body, quantitatively the most important site of the mucosal IgA production is the gastrointestinal tract because of its vast surface area (Crabbe and Heremans, 1966). Stephans and colleagues (1983a) found that nasopharyngeal organ cultures contain a large population of highly reactive, terminally differentiated B lymphocytes that might be an important source of upper respiratory tract IgA. Comparison of the molecular properties of IgA, such as subclass distribution and molecular forms in serum and external secretions and of IgA-producing cells in the bone marrow and mucosal tissues suggests a relative independence of the serum and mucosal IgA systems (Mestecky and Russell, 1986).

There is a characteristic difference in the subclass distribution of IgA to certain antigens between the two systems. Considerable information now exists on human IgA responses to infection with a variety of microorganisms or to immunization with purified antigens (Mestecky *et al.*, 1989). Studies on serum IgA1 and IgA2 to protein antigens (Russell *et al.*, 1986) and LPS (Moldoveanu *et al.*, 1987) indicated that in the serum of healthy individuals, most are of the IgA1 subclass. In contrast, salivary (Brown and Mestecky, 1985) and colostral (Ladjeva *et al.*, 1989) IgA1 and IgA2 to various antigens display characteristic patterns of distribution, though with considerable individual variability. In the majority of subjects, sIgA to protein antigens are of the IgA1 subclass, while those reactive with polysaccharide, lipopolysaccharide and lipoteichoic acid are predominantly of the IgA2 subclass.

The host defence mechanisms are not fully developed in infants. At birth, sIgA is essentially absent from the oral cavity (Cripps *et al.*, 1987). Shortly after birth IgA appears in whole saliva and its concentration increases as the infant develops (Alaluusua, 1983; Mellander *et al.*, 1984; Smith *et al.*, 1987).

It has been documented that pIgA is more effective in viral neutralization than corresponding mIgA (Taylor and Dimmock, 1985). In addition, various receptors found on many different cell types such as phagocytes, T cells and hepatocytes bind pIgA more effectively than mIgA (Mestecky and McGhee, 1987), and this might be reflected in greater functional activity of pIgA.

1.6.3 The role of IgA in local immune protection

It is generally accepted that sIgA antibodies act as a first line of defence of mucosal surfaces principally by simple binding to antigens (Hanson and Brandtzaeg, 1988). *In vivo* coating of oral bacteria with sIgA can be directly demonstrated by immunofluorescent staining (Brandtzaeg *et al.*, 1968). Human sIgA is effective in cross-linking antigens (Newcomb and Sutoris, 1974) and shows better agglutinating properties than monomeric antibodies (Newcomb and De Vald, 1969). Its primary function might be to aggregate particulate antigens. In addition, it can protect the host by binding to microorganisms or their products. Such a simple function might explain neutralization of bacterial toxins (Kaur *et al.*, 1972) and inhibition of virus. The coating and clumping of bacteria might inhibit their adherence to epithelial cells (Fubara and Freter, 1972; Hanson *et al.*, 1987). The polymeric nature of sIgA provides not only increased avidity for antigens but also enhanced ability to cross-link particles (Ishizaka *et al.*, 1965).

Salivary IgA to mucosal bacteria such as *E. coli, Streptococcus mitis*, or *S. salivaris* begin to appear as early as the first week of life (Smith *et al.*, 1990). In general, the

appearance of antibodies in saliva correlates with the colonization of the oral cavity by the respective microorganisms.

When microorganisms such as virus particles succeed in reaching the epithelium, they attach to the membrane through receptors complementary to structures on the epithelial cell surfaces. Binding of the antibody to the surface of an organism could prevent attachment by direct blocking, steric hindrance or induction of conformational change (Dimmock, 1984; Lachmann, 1985). Purified sIgA antibodies have been shown to prevent the attachment of bacteria to mucosal surfaces (Fubara and Freter, 1973; Svanborg-Eden and Svennerholm, 1978). sIgA can specifically inhibit cellular attachment and penetration of influenza virus in contrast to monomeric IgA and IgG neutralizing antibodies (Taylor and Dimmock, 1985).

1.6.3.1 IgA protease

sIgA is remarkably resistant to protease action by enteric and oral bacteria; however, meningococci produce a protease that specifically cleaves molecules of the subclass IgA1 into Fc and Fab fragments (Kilian and Reinholdt, 1986). The production of IgA1 protease by meningococci has been postulated to be an important virulence factor (Plaut, 1983). Bacteria that colonize the IgA-rich environment of the upper respiratory tract such as *N. meningitidis* and *H influenzae* produce IgA proteases and might cause a relative local sIgA deficiency. IgA2, unlike IgA1, is resistant to these enzymes (Killian and Reinholdt, 1986).

1.6.3.2 IgA antibodies in milk

Some of the best evidence that sIgA antibodies are protective can be found in studies of human milk. Epidemiological studies in communities where bacterial and viral intestinal infections are common indicate that infants who receive mother's milk containing antibodies to intestinal pathogens have less disease due to those organisms

than infants receiving mother's milk without the antibodies. For instance, anti-adhesin and anti-toxin antibodies against cholera seem to reduce diarrhoeal disease (Glass *et al.*, 1983). This suggests that these sIgA antibodies which are at high concentrations in many samples of human milk can passively protect the infant. For some pathogens such as rotavirus, feeding antibody-containing milk diminishes the severity of the disease (Duffy *et al.*, 1986) and might reduce the infection rate. Goldman and colleagues (1986) suggested that specific sIgA molecules, along with the other immune factors present in the milk, confer disease protection through non-inflammatory antimicrobial mechanisms.

1.6.3.3 IgA and phagocytosis

The effects of IgA and sIgA on functions of polymorphonuclear cells (PMN), monocytes, macrophages, and lymphocytes, all of which express Fc-alpha receptor, have been reported. Oral cavity PMN express more Fc-alpha receptors than blood PMN; and oral but not blood PMN phagocytose red cells coated only with sIgA. Human blood monocytes are able to kill sIgA coated gonococci by antibody-dependent cell mediated cytotoxicity (ADCC) (Vaerman, 1987).

1.6.4 Secretory IgM

Relatively small amounts of 19S IgM are found in normal secretions, but the concentration is much increased in infants and in individuals with selective IgA deficiency (Brandtzaeg, 1971; Savilahti, 1973). It was originally thought that 19S IgM antibodies in serum and secretions were identical (Brandtzaeg *et al.*, 1978); but it has been shown that IgM in secretions is associated with SC although only 60 to 70% of the molecules retain the SC after purification (Brandtzaeg, 1975). sIgM is not as resistant to proteolytic degradation as sIgA (Richman and Brown, 1977).

sIgM is increased in infancy and is the prominent secreted isotype for the first few months after birth (Glesson *et al.*, 1982) and in selective IgA deficiency (Brandtzaeg, 1971). Various antibody activities have been shown for IgM in nasal secretions (Ogra *et al.*, 1974) and salivary fluids (Mellander *et al.*, 1984; 1986a and b). Evidence from measurement of antibody activity to antigens to which infants are naturally exposed (Mellander *et al.*, 1984, 1986a) or to which they have been immunized (Carlsson *et al.*, 1985; Smith *et al.*, 1986) indicates that IgM antibody is also present in whole saliva early in life. IgM has been detected in saliva of some infants who were as young as one month of age (Glesson *et al.*, 1982; Smith *et al.*, 1989).

Hanson and colleagues (1987) have observed that IgM activity decreased as IgA antibody activity increased in infants and suggested that both isotypes were contributing to protective functions in the oral cavity. It is probable, therefore, that IgM might function like sIgA as a first line of mucosal defence. Thrane and colleagues (1991) observed more IgM than IgA producing cells during the foetal and neonatal period. This is in accordance with a preferential early salivary IgM response (Mellander *et al.*, 1986a). During this period, and up to about 2 years of age, there is a striking increase of the IgA level (Alaluusua, 1983). In the following few years very little increase seems to take place (Alaluusua, 1983; D'Amelio *et al.*, 1986); most studies agree that adult salivary IgA levels are reached late in childhood (Burgio *et al.*, 1980; D'Amelio *et al.*, 1986). Thrane and colleagues (1991) suggested that the local production of IgA increased up to 15 months, while no such increase was observed for IgM and IgG cells after 7 months.

1.6.5 Secretory IgM function

There is evidence to suggest that protection against mucosal infection in individuals with selective IgA-deficiency might be effectively provided by the compensatory local production of virus specific IgM and IgG in the intestinal and respiratory tracts

respectively (Ogra *et al.*, 1974). Gregory and colleagues (1985) found that IgA-deficient subjects had fewer carious lesions than did immunoglobulin-deficient individuals, suggesting that the salivary IgM antibodies reduced the level of caries in these individuals. Arnold and colleagues (1977) observed sIgM in the saliva of 11 out of 38 IgA-deficient patients which comprised functional antibodies directed to antigens of oral bacteria. Immunohistochemical examination of salivary glands from such patients revealed numerous IgM-containing plasma cells in locations occupied in normal individuals by IgA plasma cells (Brandtzaeg, 1975); and significantly increased levels of innate humoral factors, including lysozyme, were detected (Arnold *et al.*, 1977).

1.7 Prevention of meningococcal disease: chemoprophylaxis and vaccines

Most cases of meningococcal disease result from the transmission of a virulent strain of *N. meningitidis* from the nasopharynx of a healthy carrier to a susceptible host. Many factors facilitate susceptibility to the disease including complement (Ross and Densen, 1984) and immunoglobulin deficiencies (Hobbs *et al.*, 1967); however, the only clearly defined risk factor for the development of disease is being a close contact of an index case (Meningococcal Disease Surveillance Group, 1976a; Cooke *et al.*, 1989).

Chemoprophylaxis of contacts of cases can offer protection for a limited period of time. Only active immunization, preferably of young children who are at greatest risk, can induce long lasting immunity. Effective vaccines offer the best chance of significantly reducing the incidence of bacterial meninigitis as has been demonstrated with vaccines against type b *H. influenzae*. Prevention of infection is the only realistic method of reducing the long term morbidity and mortality of meningococcal disease.

1.7.1 Antibiotic prophylaxis

Eradication of *N. meningitidis* from the pharynx of carriers is considered important in the management of outbreaks of meningococcal disease. Prophylaxis with antibiotics is currently recommended for contacts of persons with invasive disease caused by *N. meningitidis*: members of the same house-hold (Olcen *et al.*, 1981); children in day-care centres (De Wals *et al.*, 1981); closed populations such as military recruits (Sivonen *et al.*, 1978). Household contacts of patients with meningococcal meningitis often become colonized by the microorganism (McCormrick and Bennett, 1975); and, in many cases newly acquired bacteria can lead to disease (Sanders and Deal, 1970).

Sulphonamides were effective not only in eradicating the carrier state but also in preventing meningococcal disease in military populations; however, the use of sulphonamides for chemoprophylaxis is now limited by resistance to these drugs in many areas of the world. In 1973, 57% of serogroup A, 20% of serogroup B, 75% of serogroup C and 4% of serogroup Y meningococcal isolates were resistant to sulphonamide (McCormick *et al.*, 1974). The recent outbreak in Stonehouse and Airdrie were due to sulphonamide-resistant strains (Cartwright *et al.*, 1987; Blackwell *et al.*, 1990).

Other antibiotics have been examined as alternatives to sulphonamides but with limited success. Penicillin, ampicillin and erythromycin were ineffective for eradication of nasopharyngeal carriage of meningococci (Band *et al.*, 1983); however, success has been obtained with rifampicin, minocycline and ciprofloxacin. Rifampicin is as effective as minocycline (Weidmer *et al.*, 1971; Shapiro and Wald, 1980). It eliminates meningococci from the nasopharynx and protects against recolonization (Sivonen *et al.*, 1978). Several studies have documented the emergence of resistant strains of meningococci following treatment with rifampicin (Weidmer *et al.*, 1971). In different studies, up to a 12% of the isolates in the nasopharynx were resistant to this

antibiotic (Weidmer *et al.*, 1971). A recent study in Gloucestershire found chemoprophylaxis with rifampicin to be 96% effective in eradicating nasopharyngeal carriage, but a high secondary attack rate was noted (Stuart *et al.*, 1989). The secondary cases can be defined as disease in a household contact that begins more than 24 hours and less than 30 days after the index case in the household is hospitalized (McCormick and Bennett, 1975).

Ciprofloxacin, a carboxyquinolone antibiotic, has been demonstrated to be effective in adults in eradicating nasal carriage even after a single oral dose (Gaunt and Lambert, 1988). A single intramuscular dose of ceftriaxone proved to be superior to rifamipcin in eradicating carriage of serogroup A meningococci in Saudi Arabia (Schwartz *et al.*, 1988). Further studies on the protective efficacy of this agent are required before its routine use can be recommended.

1.7.2 Vaccines

1.7.2.1 Development of polysaccharide vaccines for serogroup A and serogroup C

In the late 1960s when the usefulness of sulfadiazine as a prophylactic agent began to decline and other antibiotics proved to be inadequate substitutes, purified carbohydrate antigens derived from the capsules of *N. meningitidis* were developed as vaccines against serogroups A and C. Their safety and efficacy in older children and adults have been demonstrated in a number of controlled field trials and epidemic situations (Erwa *et al.*, 1973; Greenwood and Wali, 1980; Peltola, 1978). Although there are 13 different meningococcal capsular serogroups (Apicella, 1990), serogroups A, B and C are responsible for approximately 90% of the cases of meningococcal meningitis (Gotschlich *et al.*, 1977). A tetravalent vaccine composed of serogroup A, C, W135 and Y has proved to be safe and in humans (Cadoz *et al.*, 1985) and is currently in use.

Unfortunately, only the serogroup A polysaccharide was shown to be an effective immunogen in infants. Protection by vaccination against disease due to serogroup C organisms has not been demonstrated in infants below 2 years of age (Frasch, 1983). Since the peak incidence of meningococcal disease occurs among children less than two years of age, alternative vaccines are necessary to induce protective immunity to serogroup C vaccine into the age group at greatest risk.

The immunity gained after recovery from infection by encapsulated bacteria, in terms of the polysaccharide antigens, differs from that generated by immunization with purified capsular polysaccharide vaccines (Jennings, 1983). Generally, the immune response to purified polysaccharides is thymus-independent with production of IgM antibodies; and, no enhancement of the response is observed after subsequent immunization. There are exceptions in which a booster response can be induced in young children with meningococcal serogroup A polysaccharide (Gotschlich *et al.*, 1978), and high levels of IgG antibodies can be induced in mice with the serogroup C polysaccharide (Moreno and Esdaile, 1983). Capsular polysaccharide cannot induce consistent, boostable high titre antibody responses; and, they cannot prime by themselves for IgG memory response (Anderson *et al.*, 1985).

By comparison with adults, infants respond very poorly to polysaccharide vaccines (Gotschlich *et al.*, 1977; Peltola *et al.*, 1977); the antibodies produced are almost exclusively of the IgM isotype, and the immune response is not enhanced by subsequent vaccination. In infants, due to the nature of their immune responses, these polysaccharide vaccines are only of marginal benefit (Robbins, 1978).

The nature of these different responses in humans has been investigated by studies in children on the cellular basis of the immune response to polysaccharide. In healthy human infants, the antibody response to several polysaccharide antigens are delayed in ontogeny (Smith *et al.*, 1973; Borgono *et al.*, 1978). Precursers of B cells expressing

specificity for different polysaccharide antigens such as α-1,3-dextran (Howard and Hale, 1976) and levan (Bona *et al.*, 1979) have been shown to appear late in ontogeny, whereas precursers to some proteins have been detected on the day of birth.

Polysaccharides have traditionally been characterized as T-independent antigens, capable of activating B cells without T cell help, producing mainly IgM antibody. Young children make little or no antibody to such T-cell independent antigens (Anderson and Betts, 1989). To overcome this problem, polysaccharides have been conjugated to proteins. Schneerson and colleagues (1980) adopted this approach and produced conjugates of the capsular polysaccharide of *H. influenzae* type b with several proteins. This preparation proved to be significantly more immunogenic than the polysaccharide alone when injected into immature animals and humans (Schneerson *et al.*, 1986).

The serogroup A meningococcal vaccine is immunogenic in adults and children, although antibody responses in children less than two years of age are significantly lower than in older children (Goldschneider *et al.*, 1973). The duration of the immunity is also age related; only older children demonstrate evidence of vaccine derived immunity three years after primary immunization (Kayhty *et al.*, 1980). In contrast to polyribosyl phosphate (PRP) of type b *H. influenzae* and other polysaccharide vaccines, immune responses can be elicited to the serogroup A meningococcal vaccine in children as young as 6 months of age and a booster response can be demonstrated in children who have been immunized (Goldschneider *et al.*, 1973; Gold *et al.*, 1975; Kayhty *et al.*, 1980). Serogroup A meningococcal polysaccharide-protein conjugate vaccines are under development; these might provide higher level and longer lasting immunity.

The serogroup C meningococcal polysaccharide vaccine elicits a weaker antibody response than the serogroup A vaccine. As with other polysaccharide vaccines, it is

poorly immunogenic in infants and children less than two years of age (Goldschneider *et al.*, 1973). The duration of the antibody response is also age related, with a faster decline in antibody levels compared with the serogroup A vaccine (Gold *et al.*, 1979). No booster response is observed (Goldschneider *et al.*, 1973; Gold *et al.*, 1975), and there is some suggestion of immunologic tolerance. Infants immunized at a young age can have lower antibody responses after the booster dose than they did after their primary immunization (Gold *et al.*, 1975).

1.7.2.2 Problems associated with development of vaccines for serogroup B capsular antigen

There has been a spectacular lack of success in producing an effective vaccine against disease caused by serogroup B meningococci. In early trials, the high molecular weight serogroup B capsular polysaccharide proved to be very poorly immunogenic even in adults (Wyle *et al.*, 1972). This has been attributed to immune tolerance induced by the close structural similarity between the polysaccharide and antigenically cross-reacting oligosaccharides found in human and animal foetal brain (Finne *et al.*, 1983). The serogroup B polysaccharide primarily induces low-avidity IgM antibodies with little or no bactericidal activity in conjunction with human complement (Zollinger and Mandrell, 1983).

1.7.2.3 Group B polysaccharide-OMP complexes

Much research has been carried out on the major OMPs of meningococci as potential vaccines. Since the response to OMP vaccines is serotype dependent, an effective vaccine would have to contain a number of the most common serotypes. The outer membrane proteins of serogroup B meningococci induce anti-meningococcal antibodies after disease or vaccination (Griffiss *et al.*, 1987a). A vaccine effective against multiple serogroups, especially serogroup B and C, might need to contain outer membrane proteins from relatively few serotypes. Much of the work on OMP

vaccines has been based on serotype antigens found among strains causing outbreaks in Northern Europe and America. Recent studies suggest that the majority of strains isolated from patients with meningococcal disease in Greece are non-typable. Vaccines based on serotype or subtype antigens need to be tailored for specific areas based on epidemiological monitoring (Tzanakaki *et al.*, 1993).

Serogroup B polysaccharide non-covalent complexed with meningococcal OMPs has been prepared in several laboratories (Zollinger *et al.*, 1979; Frasch and Peppler, 1982; Moreno *et al.*, 1985). These vaccine are now being tested in Cuba (Sierra *et al.*, 1991), Chile (Zollinger *et al.*, 1991) and Norway (Rosenqvist *et al.*, 1991). Such a vaccine is a mixture of different components, they contain class 1, class 2/3, class 5 OMPs and LOS. The majority of bactericidal response generated by these vaccines is induced by the class 1, class 2/3 proteins and LOS (Apicella, 1991).

1.7.2.4 Enhancement of immune response to the B capsular polysaccharide

Another approach is to enhance the immune response to the group B polysaccharide either by physical manipulation (Zollinger *et al.*, 1979; Frasch and Peppler, 1982; Moreno *et al.*, 1985) or by chemical modification creating synthetic epitopes capable of modulating the immune response in such a way as to produce enhanced levels of cross-reactive antibodies specific for B polysaccharide (Jennings *et al.*, 1986). Sarvamangala and colleagues (1991) have developed a vaccine consisting of the synthetic B polysaccharide conjugated with tetanus toxoid. So far the vaccine has been tested only in mice.

1.7.7.5 Recommended uses of vaccine

The use of capsular polysaccharide as an immunoprophylactic agent against human disease caused by encapsulated bacteria is now firmly established, mainly for military

personnel. Licensed vaccines are now available in Britain against serogroups A and C (Meningivac A & C or ACVax); immunization is recommended for local outbreaks and contacts of patient with disease due to serogroups other than B (Department of Health, 1990).

Routine vaccination of the civilian population in developed countries in not currently recommended because the risk of infection is low and most of the endemic disease occurs in young children. Vaccination is advised to control outbreaks due to meningococcal serogroups covered by the available vaccines. Routine vaccination is recommended for travellers to countries recognized as hyperendemic with periodic epidemic meningococcal disease, such as the meningitis belt of Africa or areas where recent epidemics have occured such as Saudi Arabia and Nepal. Vaccination of individuals with a deficiency in one of the terminal complement components or with properdin deficiency might be effective in preventing disease.

The use of serogroup C vaccine was found to reduce carriage rate by 30-50% (Gotschlich *et al.*, 1969b). In another study, a vaccination trial in Africa found serogroup A polysaccharide vaccine had no effect on the carriage rate (Blakebrough *et al.*, 1983).

Despite the many advantages of capsular polysaccharides as human vaccines, one serious limitation of their general use is the poor immune response to these purified polysaccharides in infants (Robbins, 1978; Jennings, 1983). Determination of the host parasite interactions that enhance susceptibility of individuals to the disease might identify the population that should be immunized. Prevention of many of the deaths and disability caused by meningococcal disease will depend on the efficacy of the vaccines in infants.

1.8 Blood groups, secretor status, and susceptibility to infectious agents

1.8.1 Blood groups and diseases

There is a large body of epidemiological evidence for associations between ABO blood groups and secretor status with susceptibility not only to infectious disease but also to other conditions (Mourant, 1989). Examples of the reported association for infectious disease and ABO blood groups are given in Table 1.2 and that for secretor status in Table 1.3. Many of the associations between ABO blood groups and diseases were severely criticised by Wiener (1970) who stated that if an hypothesis could not be formulated to explain the findings, they were most likely due to chance. Associations between ABO blood groups and infectious diseases were excluded from these criticisms as many micro-organisms have antigenic components cross-reactive with ABH antigens.

The ABO blood group system was first described by Landsteiner in 1900. Epstein and Ottenberg suggested that the ABO blood groups were inherited characteristics (reviewed by Race and Sanger, 1975) and later it was demonstrated by von Dungern and Hirszfeld in 1910 that the blood group of an individual was inherited as a Mendelian trait in a dominant pattern (reviewed by Mourant *et al.*, 1978). The ABO groups are inherited through multiple alleles at one locus. Many additional blood groups were discovered after the ABO system, *e.g.*, Kell, Duffy, M, N, S, I, P and Lewis.

It has long been known that the ABH blood group determinants are not confined to red cells but can be found in many secretions and on numerous cells of the human body. Similar structures are widely distributed in many animals, some plants and many bacteria. Springer (1970) demonstrated that Gram-negative bacteria express substances which are similar to the A, B and H antigens of human.

Table 1.2: Association between ABO blood groups and susceptibility to infections

Infectious agent/disease	Blood group associations	References
Respiratory tract		
Influenza A	O	Potter, 1969
M. tuberculosis	B	Viskum, 1975
S. pyogenes (Group A)	not O	Haverkorn, and Goslings, 1969
S. pneumoniae	not B	Reed *et al.*, 1974
Oral cavity		
Candida albicans (carriage)	O	Burford-Mason *et al.*, 1988
Periodontal disease	O and AB	Pradhan *et al.*, 1971
Gastrointestinal tract		
E. coli	B	Socha *et al.*, 1969
Salmonella and *E. coli*	B and AB	Robbinson *et al.*, 1971
Vibrio cholerae	O	Barua and Paguio, 1977
Urinary tract		
E. coli	B	Cruz-Coke and Paredes, 1965
	B/AB	Kinane *et al.*, 1982
Genitourinary Tract		
N. gonorrhoeae	B	Foster and Labrum, 1976
	no association	Johnson *et al.*, 1983
Chlamydia trachomatis	B	Blackwell (unpublished observation)
Blood borne infections		
Malaria	A	Gupta and Chaudhuri 1980
Coccidioides immitis	B	Derensiski *et al.*, 1979

Table reproduced from Blackwell (1989) with modifications.

Table 1.3: Association between secretor status and susecptibilty to infections

Infectious agent/disease	Secretor status associations	References
Respiratory tract		
Respiratory Syncytial Virus	secretors	Raza *et al.*, 1991
Rhino virus	secretors	Raza *et al.*, 1991
Echo virus	secretors	Raza *et al.*, 1991
Influenza A and B	secretors	Raza *et al.*, 1991
Parainfluenza virus	none	Raza *et al.*, 1991
S. pyogenes (Group A)	non-secretors	Haverkorn and Goslings, 1969
S. pneumoniae	non-secretors	Blackwell *et al.*, 1986a
N. meningitidis	non-secretors	Blackwell *et al.*, 1986a
H. influenzae (type b)	non-secretors	Blackwell *et al.*, 1986b
Oral cavity		
Candida albicans carriage	non-secretors	Burford-Mason *et al.*, 1988 Aly *et al.*, 1992
C. albicans infections	non-secretors	Thom *et al.*, 1989 Aly *et al.*, 1991
Caries	non-secretors	Holbrook and Blackwell, 1989
Periodontal disease	none	Pradhan *et al.*, 1971
Gastrointestinal tract		
Vibrio cholerae	non-secretors	Chaudhuri and Das Adhikary, 1978
Urinary tract		
E. coli	non-secretors	Kinane *et al.*, 1982
Genitourinary Tract		
Neisseria gonorrhoeae	none	Johnson et al., 1983
Chlamydia trachomatis	non-secretors	Blackwell (unpublished observation)
HIV (heterosexual transmission)	secretors	Blackwell *et al.*, 1991
HIV (heterosexual transmission)	none	Blackwell *et al.*, 1991
HIV (Intravenus drug users)	none	Blackwell *et al.*, 1991

Table reproduced from Blackwell (1989) with modifications.

1.8.2 Distribution of secretor status

The majority of individuals have the water-soluble glycoprotein form of their ABO blood group antigens in their body fluids (saliva, urine and ovarian cyst fluid). Non-secretor individuals do not express their ABO blood group antigens in body fluids. Because the secretor gene (*Se*) is inherited in a Mendelian dominant pattern, the predicted proportion of these two phenotypes within a population is secretors 75-80% and non-secretors 20-25%. This ratio can vary widely in different ethnic groups and some geographically isolated populations (Mourant *et al.*, 1978).

Epidemiological evidence from different studies in the United Kingdom, Iceland and Nigeria suggest that there is an association between non-secretion and susceptibility to invasive disease caused by meningococci, pneumococci and type b *H. influenzae* (Blackwell *et al.*, 1986a; 1986b). In areas where there have been prolonged outbreaks of meningococcal disease and where the secretor status of the population has been determined (Northern Nigeria, Iceland), there are unusually high proportions of non-secretors (Blackwell *et al.*, 1990). In the United Kingdom a similar pattern was found in Stonehouse (Glouscestershire) (Blackwell *et al.*, 1989b) and Plymouth (Blackwell and Weir, 1990) where there have been prolonged outbreaks due to the B:15:P1.7,16 strains and among a school population in which an outbreak of meningitis due to a B:4:P1.15 strain occurred (Blackwell *et al.*, 1990).

1.8.3 The H, Lewis and secretor genes

The secretor (*Se*) gene also affects expression of the Lewis (*Le*) blood group antigen. Non-secretors can only express Lewis^a (Le^a) while secretors express Lewis^b (Le^b) predominantly and variable amounts of Le^a. The Le^a in non-secretor saliva was first identified by Japanese workers in 1939 (for review see Race and Sanger, 1975). In 1946 Mourant described Le^a which he had detected on red cells by the use of human agglutinating sera. Anti-Le^b was described by Andersen (for review see Race and

Sanger, 1975). Unlike the ABO antigens which are part of the structure of red cells, Le^a or Le^b antigens are adsorbed from the plasma (see Race and Sanger, 1975).

The *Se*, *H* and *Le* genes have been mapped to the same linkage group of chromosome 19 (see Watkins, 1980). There is active gene *H* and a silent gene *h*. The H-deficient phenotype (h/h) is refered to as Bombay phenotype. Most of these H-deficient individuals also lack the H determinants in saliva. The (*Se*) gene also has an active allele *Se* and a silent allele *se*. An individual homozygous for the (*SeSe*) or heterozygous (*Sese*) is a "secretor"; whereas, an individual homozygous for the silent (*sese*) is a "non-secretor".

1.8.4 Structures of the ABO and Lewis blood groups

The ABO blood group determinants are carried on macromolecules, mainly glycoproteins in secretions and glycolipids and glycosphingolipids on the cell surfaces. Blood group molecules consist of a peptide or lipid backbone to which relatively short carbohydrate chains are attached. It is generally assumed that the synthesis of carbohydrate structures occurs in the endoplasmic reticulum and Golgi complex by the stepwise addition of single sugars, each addition being catalysed by a relatively specific glycosyl transferase (Dawson, 1978). Most transferases are specific for both the sugar they transfer and the acceptor molecule. Some transferases such as that coded for by the *Le* gene, can transfer the same monosaccharide to several different acceptors leading to the formation of Le^a and Le^b (Figure 1.2).

1.8.4.1 Precursor chains

The backbone consists of carbohydrate chains, the terminal groupings of which are responsible for the specificity. The actual specificity of the molecule is determined by the sugar occurring at the non-reducing end of the chain. Four types of precursor

Table 1.4: Main types of ABH active oligosaccharide

Type	Structure
Type 1	Galβ1→3GlcNAcβ→R
Type 2	Galβ1→4GlcNAcβ→R
Type 3	Galβ1→3GalNAcα→R
Type 4	Galβ1→3GalNAcβ→R

Gal = D-galactose
GlcNAc = N-Acetylglucosamine
GalNAc= N-Acetylgalactosamine

chains that carry the ABH determinants have been described (Rege *et al.*, 1963, Donald, 1981, Bremer *et al.*, 1984) Table 1.4.

Type 1 precursor is synthesized in epithelia (Rege *et al.*, 1963). It constitutes the main carrier of ABH and Lewis epitopes in body fluids and secretions. Because they are adsorbed from the plasma, they are present on the surface of the erythrocytes. Type 2 precursor is the main type found on erythrocytes and coexists with Type 1 precursor on epithelia (Rege *et al.*, 1963). Both Type 1 and Type 2 chains can occur as branches on a single carbohydrate chain. Type 3 and Type 4 precursor chains have been only recently discovered (Takasaki *et al.*, 1978; Donald, 1981). Their biosynthetic regulation and distribution is not yet fully understood (Clausen *et al.*, 1986).

1.8.4.2 ABH antigens

The terminal sugar responsible for A specificity is N-acetyl-galactosamine attached to C-3 of the terminal galactose of the H structure, while in the same position D-galactose determines the B specificity and L-fucose is the sugar associated with H the antigens of blood group O. The Type 1 precursor chain is fucosylated by the product of the *Se* gene, an α1-2 fucosyltransferase to form H Type 1 determinants (Watkins *et al.*, 1988). H Type 1 acts as a substrate for A or B glycosyltransferases to give rise to their respective antigens. The *H* gene codes for production of a fucosyltransferase enzyme which adds L-fucose to the C2 position of the terminal sugar residue of Type 2 chains. The steps in synthesis of the structures of H (Lloyd *et al.*, 1966), A and B (Painter *et al.*, 1965; Lloyd *et al.*, 1966) determinants based on Type 1 and Type 2 chains are shown in Figure 1.1.

1.8.5 Interaction of the products of the *A*, *B*, *H*, *Se* and *Le* genes

Once the *H* gene encoded transferase has added L-fucose to Type 2 chains, the *A* or *B* gene-specified products can act to add sugars to the chains that carry H determinants. Cells of O individuals express the unmodified structure of the H antigen. When both

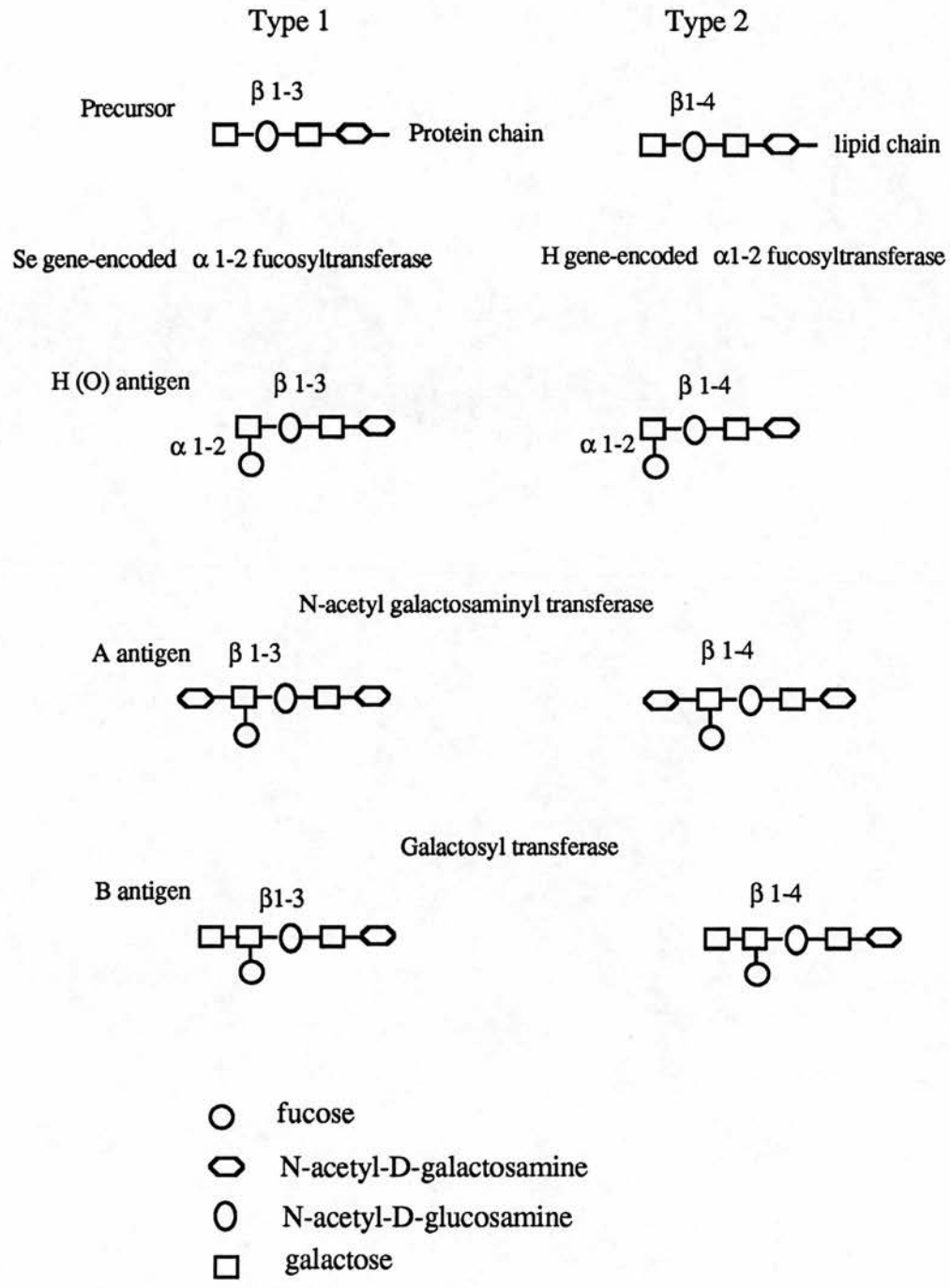

Figure 1.1: Production of A, B and H (O) antigens from precursor Type 1 and 2 chains. The enzymes facilitating conversion of antigens are shown. Note that H Type 1and H Type 2 are substances for N-acetyl galactosaminyl transferase and/or galactosyltransferase.

H and A transferases are present, the precursor chains are converted first to H and then to A. A similar conversion to B occurs with H and B transferases (Figure 1.2).

The presence of the *Se* gene means that individuals with *hh/SeSe or hh/Sese* will express Type 1 chain based blood group antigens in their secretions but will have no ABH antigens on their red cells. Such individuals are refered to as para-Bombay phenotype.

A and B substances cannot appear in saliva without the prior conversion of the Type 1 precursor substance into H. This conversion does not take place in the absence of the *Se* gene. The failure to secrete A and B substances depends on the absence of the requisite H Type 1 structure and not on the absence of the products of the A and B genes.

The production of the Le determinants is not dependent upon the *Se* gene product; therefore, Le^a substance is produced whether or not the *Se* gene is present. Although there are two antigens, Le^a and Le^b, there is only one *Le* gene, which encodes an α1-3/4 fucosyltransferase. The enzyme adds L-fucose to the C-4 position of the subterminal N-acetylglucosamine of the H Type 1 or precursor Type 1 chain (Oriol *et al.*, 1986). The presence of Lewis antigens in secretions and on cell surfaces depends on the expression of the *Le* gene and not on the *Se* gene. Individuals who do not express any Lewis determinants on their red blood cells or in secretions are known as Lewis-negative (Le^{a-b-}).

Since the Lewis antigens are not fully developed at birth, haemolytic disease of new born due to anti-Le^a is almost unknown. Le^a determinants are present on the cells of 80-90% of infants at around two months of age (Issit, 1986). Infants express detectable amounts of Le^a, because Lewis enzyme is more active than *Se* gene product. As the *Se* enzyme becomes more active, the levels of Le^a drop with the appearance of Le^b. These interactions are summarized in Figure 1.2.

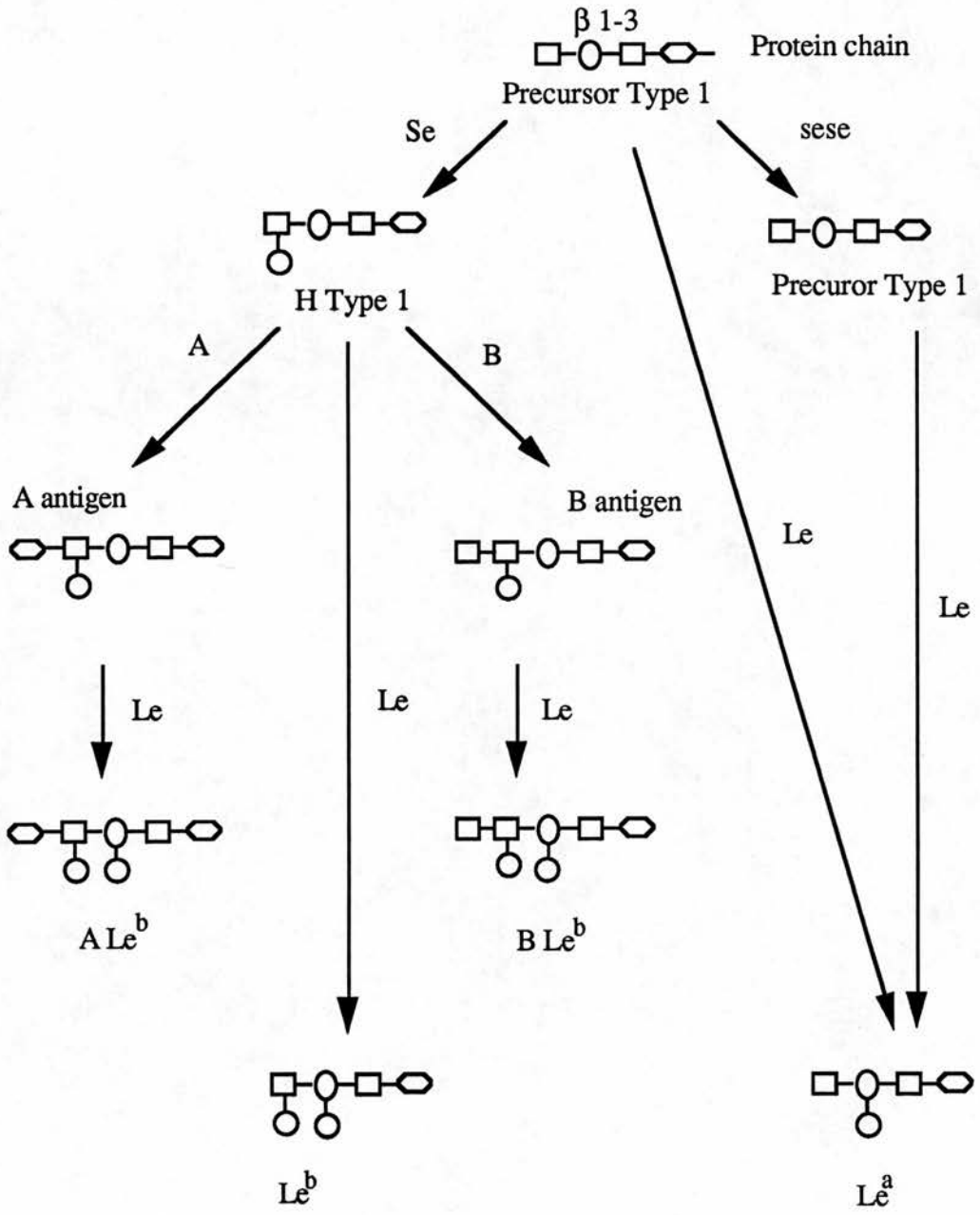

Figure 1.2: Production of H, A, B and Lewis antigens in secretions. Note that the α 1-2 fucosyltransferase (*Se* gene-encoded) and the α1-4 fucosyltransferase (*Le* gene-encoded) compete for the precursor Type 1 chain. The α1-4 fucosyltransferase also competes with the A and B glycosyltransferasees (see text). Symbols as in figure 1.1.

1.8.6 Mechanisms underlying susceptibility of non-secretors to bacterial infections

1.8.6.1 Factors affecting colonization

Table 1.5 lists examples of blood group antigens that act as receptors for microorganisms (Blackwell, 1989). The increased proportion of non-secretors among carriers of some potentially pathogenic bacteria and yeasts might be attributed to the Lewis^a antigen acting as one of the host cell receptors for some organisms (Blackwell, 1989; May *et al.*, 1989; Rahat, 1990, Tosh and Douglas, 1991; Aly, 1992; Saadi *et al.*, 1993). Saadi and colleagues (1993) found that buccal epithelial cells (BEC) obtained from non-secretors bound toxigenic strains of staphylococci in greater numbers compared with BEC from secretors. Pretreatment of epithelial cells with either monoclonal anti-Lewis^a or anti-precursor type 1 antibodies significantly reduced bacterial binding. The attachment of bacteria was positively correlated with the amount of Lewis^a antigen detected on cells of secretors and non-secretors. A membrane protein of 67 K. dalton has recently been isolated by affinity purification with synsorb Le^a (Saadi *et al.*, submitted for publication). It has been suggested that the expression of Lewis^a antigen among young infants might enhance their colonization by staphylococci. The role of Lewis^a antigen in body fluids on bacterial inhibition needs to be further investigated.

1.8.6.2 Immune responses, blood groups and secretor status

1.8.6.2.1 Cholera and ABO association

Group O individuals are at greater risk of cholera than individuals of any other ABO blood group; blood group AB individuals are at lowest risk (Barua, and Paguio, 1977; Chaudhuri, 1977; Levine *et al.*, 1979; Glass *et al.*, 1985). The work of Clemens and colleagues (1989) supported these findings, but indicated that these relationships apply

Table 1.5: Blood group antigens that are receptors for microorganisms

Microorganisms	Blood group receptors	References
E. coli uropathogenic strains	P	Kallenius *et al.*, 1980
	M	Vaisanen *et al.*, 1982
	N	Jann *et al.*, 1988
Septicaemia and neonatal meningitis	S	Korhonen *et al.*, 1984
H. influenzae type b	Anton	Van Alphen *et al.*, 1986
S. aureus	Le^a	Saadi *et al.*, 1993
Candida		May *et al.*, 1986
N. meningitidis		Raza *et al.*, unpublished observation
Plasmodium knowlesi	Duffy	Miller *et al.*, 1975

Table reproduced from Blackwell (1989) with modifications

only to El Tor cholera. The secretion of blood group substances has not consistently correlated with the risk of cholera (Chaudhuri and DasAdhikary, 1978; Glass *et al.*, 1985). As part of a large field trial, Clemens and colleagues (1989) investigated the relationship between efficacy of the vaccine for cholera and ABO blood groups. The protection induced by the vaccine to severe cholera was lowest for group O vaccinees compared with other blood groups. It was suggested that this might reflect a less effective immune response to the vaccine in group O participants. No hypothesis as to the mechanisms involved was proposed.

1.8.6.2.2 Immune responses and secretor status

The differences in humoral immune responses associated with secretor status have been investigated by a number of workers for their possible contributions to prevention of infection and to explain the increased susceptibility of non-secretors to infection. The lower immunoglobulin levels found in non-secretors compared to secretors were used to explain the increased susceptibility of non-secretors to rheumatic fever and rheumatic heart disease (Glynn *et al.*, 1956, Clarke *et al.*, 1960). Streptococcal tonsillitis was observed more frequently in non-secretors than in secretors (Chipail *et al.*, 1966). Haverkorn and Gosling (1969) summarized the studies on secretor status and group A streptococcal disease and carriage. Group A *S. pyogenes* was isolated more frequently from non-secretors than secretors, indicating that secretor status is associated with carriage of these bacteria. The initiation of disease following colonization could be influenced by the ability of an individual to generate an immune response to the bacteria. Lower levels of immunoglobulins found in non-secretors compared with secretors might result in increased susceptibility to streptococcal infection.

Grundbacher and Shreffler (1970) found that the level of the anti-B isoantibodies in sera of secretors was significantly higher than those ofnon-secretors; subsequently, other studies found that non-secretors had lower levels of total salivary IgA

(Waissbluth and Langman, 1971) and serum IgA (Grundbacher, 1972). Secretors had higher levels of serum IgG compared with non-secretors, but the differences were not statistically significant. The authors suggested this might be due to the small number tested (Waissbluth and Langman, 1971).

Blackwell and colleagues (1987) studied the association between secretor status and immunoglobulin levels among women who had been followed for 20 years because of problems with recurrent urinary tract infections. Non-secretor women in this group had significantly higher levels of total serum IgA than the secretors. The levels of total serum IgA among non-secretors who had improved over the 20 years period were higher than those found for secretors who had improved. No differences were found between these immunoglobulin levels for secretors and non-secretors who had not improved or in whom there was no change in the number of infections during the second decade compared with the first decade.

Antibody levels and the carriage of *N. meningitidis* was studied by Blackwell and colleagues (1989a). There was no difference in the levels of IgA in the saliva of secretors compared with non-secretors from whom no neisseriae were isolated. Compared with non-carriers, carriers had significantly higher levels of salivary IgA. Both secretor and non-secretor carriers of *N. lactamica* had salivary IgA levels comparable to those of non-carriers. Similar results were obtained when patients with spondyloarthropathies; no difference in total serum or salivary IgA between secretors and non-secretors was found (Shinebaum *et al.*, 1987).

These conflicting results reported for the differences in immunoglobulin levels between secretors and non-secretors might be due to effects of environmental factors or differences in techniques for assessment of antibody levels. Most of these studies measured total antibodies not specific antibodies for the bacteria under investigation. Barton and colleagues (1990) found that smokers have higher levels of IgM and lower

IgA in saliva. Most studies have been limited to serum or saliva (Grundbacher and Shreffler, 1970; Grundbacher 1972; Blackwell *et al.*, 1986; 1989a). For those studies in which both serum and saliva were used, most did not measure all three isotypes (IgA, IgM and IgG) (Blackwell *et al.*, 1986a; 1989a).

The choice of saliva is important and it is limited to either whole saliva or parotid saliva for practical reasons. Many studies that examined saliva did not describe which type of saliva was used. Unstimulated whole saliva is easy to collect, has a good flow rate, and contains only small amounts of serum derived from crevicular fluid (Brandtzaeg *et al.*, 1970; Lindstrom and Folke, 1973; Izutsu *et al.*, 1985). Mechanically stimulated whole saliva contains increased contamination with crevicular fluid. The problem with stimulated saliva obtained by chewing paraffin gum is that the gum absorbs immunoglobulins from saliva (see Barton, 1992). Parotid saliva contains lower concentrations of ABH antigens and is difficult to collect.

The choice of technique to measure the antibodies in serum and saliva is important; however, most studies measured total antibodies in serum and saliva by the SRID technique. A small error in such a measurement can result in a large error in the determination of the immunoglobulin concentration. A more sensitive technique would be particularly useful for the measurement of immunoglobulin isotypes which are present in lower concentrations.

The total amount of sIgA has often been difficult to estimate due to the presence of monomeric IgA and free SC together with sIgA in exocrine secretions. It is difficult to find a proper standard to use as a reference for the commonly employed immunodiffusion assay (Brandtzaeg *et al.*, 1970; Wadsworth and Hanson, 1976). A standard of 7S IgA preparation results in very inaccurate measurement of sIgA (Tomasi and Bienenstock, 1968). The use of a correction factor (1.4) is helpful (Brandtzaeg *et*

al., 1970) since IgA is present in the secretions predominantly in a 11S form which diffuses more slowly than the 7S form.

1.9 Aims of the study

This study examines antibodies to meningococci with reference to mucosal colonization with these bacteria and smoking, a factor associated with carriage of meningococci (Blackwell *et al.*, 1990; 1992a) and implicated in immunoglobulin levels in parotid saliva (Barton *et al.*, 1990). This was the first study designed to examine any functional differences in secretory and serum antibodies of secretors and non-secretors. The objectives of the study were:

(1) to develop a sensitive, precise and accurate ELISA method for assessing total immunoglobulins (IgA, IgG and IgM) and those isotypes specific for *N. lactamica* and *N. meningitidis* in serum and saliva;

(2) to determine if there are differences in the immunoglobulin levels of secretors and non-secretors that could contribute to the apparent increased susceptibility of non-secretors to meningococcal disease;

(3) to investigate whether there are differences in secretory IgA or IgM levels of secretors and non-secretors that might affect attachment of meningococci to epithelial cells;

(4) to determine if there are differences in bactericidal activity against pathogenic and non-pathogenic strains of *Neisseria* in the sera of secretors and non-secretors.

General materials and methods

2.1 Subjects

2.1.1 Staff and pupils of Airdrie Academy

Sera and unstimulated whole saliva specimens (357) were obtained from members of the staff and pupils (most within the 12-18 years age group) of a school in which there was an outbreak of meningococcal disease due to a serogroup B, serotype 4, subtype P1.15 sulphonamide resistant strain (B:4:P1.15) of *N. meningitidis*. Carriage of meningococci, secretor status, ABO and Lewis blood group antigens in this population were reported previously (Blackwell *et al.*, 1990). Informed consent to participate in the survey was obtained from parents or guardians of the children.

Sera and saliva examined included samples from:

1) students and staff who were carriers of *N. meningitidis* ;
2) students and staff who were not carriers matched as closely as possible for age, sex, and class or duties within the school if a staff member.

2.1.2 Subjects for binding assay

Unstimulated whole saliva and buccal epithelial cells (BEC) were collected from healthy students and members of staff from the Department of Medical Microbiology, University of Edinburgh. The subjects were matched for age, sex and blood type when a comparison was made.

2.2 Saliva and serum samples

2.2.1 Collection of saliva

Unstimulated whole saliva specimens from pupils and staff were transported to Edinburgh within six hours of collection and stored at -20 °C.

Unstimulated whole saliva was also collected from colleagues within the Department of Medical Microbiology and centrifuged at 1000 g for 20 min. The supernatant was collected and stored at -20 °C until used. The secretor state of individuals was determined by haemagglutination inhibition assays with saliva (Mollison, 1983) and ELISA for H (Rahat, 1990) or Lewis antigens (Raza *et al.*, 1991).

2.2.2 Blood specimens

Blood samples were collected and placed in EDTA anti-coagulant tubes. The plasma was separated and stored at -20 °C within 24 h of collection. Each was assayed for ABO blood groups by agglutination with monoclonal anti-A and anti-B antibodies Scottish National Blood Transfusion Service (S.N.B.T.S.) and for Lewis blood groups by tube agglutination with monoclonal anti-Le^a and anti-Le^b antibodies (S.N.B.T.S.) (Blackwell *et al.*, 1990).

2.3 Collection of epithelial cells

BEC from healthy individuals were collected between 0900 and 1100 h by rubbing the inside of the cheeks with cotton swabs. To remove the cells, swabs were agitated in 20 ml PBS. Cells were washed three times in D.PBS+B (300 g for 10 min) in a Sorvall RC2B centrifuge and their concentration adjusted to 2.5 x 10^5 cells/ml after determination of the number of cells microscopically with a Neubauer Improved haemocytometer. Cells from each subject were agitated vigorously to disrupt clumps prior to use in the adhesion assay.

2.4 Isolates of *Neisseria*

One strain of *N. lactamica* and five different isolates of *N. meningitidis* expressing different combination of serogroup, serotype and subtype antigens were obtained from the freeze-dried strains in the collection of the Infection and Immunity Laboratory, Medical Microbiology Department, University of Edinburgh. The outbreak strain and a B:15:P1.16 sulphonamide resistant isolate were obtained from Dr. R.J. Fallon, Meningococcus Reference Laboratory (Scotland), Ruchill Hospital, Glasgow, (Table 2.1). The isolates were characterised as *N. meningitidis* or *N. lactamica* by Gram stain, positive oxidase reaction and the utilization of sugars.

2.4.1 Modified New York City medium (MNYC)

Modified New York City (MNYC) was prepared as described by Young (1978). Difco gonococcal base was supplemented with 10% horse blood lysed with 0.5% Saponin, 2.5% yeast dialysate, 0.1% glucose, lincomycin (1.0 μg/ml), colistin (6 μg/ml), amphotericin B (1.0 μg/ml) and trimethoprim lactate (6.5 μg/ml). The plates were stored at 4 °C.

2.4.2 Incubation conditions

Standard incubation conditions, unless otherwise stated, were overnight at 37 °C an aerobic, CO_2 enriched (10%) humidified atmosphere.

2.4.3 Maintenance of cultures

All the isolates were maintained in the lyophilised state. When required, ampoules of the relevant strains were opened and reconstituted with sterile distilled water, 2-3 drops of the suspension were plated onto MNYC and the drops streaked out to obtain well isolated colonies. The plates were incubated overnight at 37 °C. Single colonies were subcultured for preparation of bacterial suspensions.

Table 2.1: Bacterial isolates

Strain	source	serogroup	serotype	subtype
N. meningitidis				
A11	patient	B	15	P1.16
A43	patient	B	4	P1.15
A41	carrier	B	4	-
A26	carrier	C	4	-
A48	carrier	NG*	4	-
N. lactamica				
LO1	carrier	NG	-	-

* = Non groupable

2.4.4 Enumeration of bacteria

An 18 h culture of bacteria was harvested from MNYC and suspended in saline. For each bacterial strain used, seven doubling dilutions and nine tenfold dilutions were made. The optical density (OD) was measured with a SP 30 spectrophotometer at 541 nm. Bacteria were also counted directly with a counting chamber (Thoma). A 20 µl drop of the ten fold dilution was plated onto MNYC, and incubated overnight at 37 °C. The average number of colony forming units (c.f.u) per 20 µl drop, for each dilution was multiplied by 50 and then by the dilution factor to calculate the number of c.f.u. The total count and viable count were correlated with the OD.

2.4.5 Standardization of bacterial concentration

Young (18 h) cultures were used for the ELISA. The bacteria were harvested with cotton wool swabs, the cell mass was suspended in saline and washed three times by centrifugation at 1000 g for 20 min. The bacteria were killed by resuspending them in formalin (2%) overnight. The killed bacteria were suspended in coating buffer and the bacterial concentration was calibrated at a spectrophotometer reading of 541 nm and adjusted to 4.8×10^9 c.f.u / ml.

2.5 Solutions for ELISA

2.5.1 Saline for harvesting of bacteria

NaCl (0.85%) in distilled water was dispensed into 100 ml bottles and autoclaved at 121°C for 15 min.

2.5.2 Phosphate-buffered saline (PBS)

PBS contained 8 mM $NaHPO_4$, 1 mM KH_2PO_4, 3 mM KCl and 0.15 M NaCl. The pH adjusted to 7.2.

2.5.3 Phosphate citrate buffer (PCB)

PCB contained 0.1 $NaHCO_3$ and 0.1 M $C(OH)(COOH)(CH_2.COOH)_2.H_2O$. The pH was adjusted to 5.0.

2.5.4 Coating buffer

Coating buffer contained 15 mM Na_2CO_3, 35 mM NaH_2CO_3, and 3mM NaN_3 (pH 9.6).

2.5.5 Washing buffer

Washing buffer was prepared by adding bovine serum albumin (BSA) (Sigma) 0.1% (w/v), and Tween-20 0.05% (v/v) to 0.01 M PBS (pH 7.2). The buffer was used for all washing procedures during the assay.

2.5.6 Blocking buffer

Blocking buffer contained 1% (w/v) BSA in 0.01 M PBS (pH 7.2).

2.5.7 Substrate solution

The substrate solution used to detect horseradish peroxidase contained 40 mg O-phenylenediamine in 100 ml of 0.1 M phosphate citrate buffer (pH 5.0) activated immediately before use by adding 40 µl H_2O_2 (30% v/v).

2.5.8 Monoclonal anti-human IgA

Monoclonal antibody to human IgA (α-chain specific) was a mouse immunoglobulin class IgG1, clone GA-112 (Sigma, Pool, Dorset, product number 1 0636). It was diluted in coating buffer for attachment to ELISA plates.

2.5.9 Monoclonal anti-human IgM

Monoclonal antibody to human IgM (μ-chain specific) was a mouse immunoglobulin class IgG2b, clone MB-11 (Sigma, product number 1 0636). It was diluted in coating buffer for attachment to ELISA plates.·

2.5.10 Standard-human-serum

Standard human serum is a stabilized mixture of sera from healthy adults used as a reference preparation for quantitative determination of immunoglobulins (Behring Dignostics, U.K., Code ORDT, number 07).

2.5.11 Sheep anti-human IgM (μ-chain specific)

The anti-human IgM serum is a pool of selected sheep sera which has been highly purified for anti-μ chain by solid-phase immunadsorption by the Scottish Antibody Production Unit (SAPU) (product code S 042-205).

2.5.12 Horseradish peroxidase (HRP) conjugated antibodies

2.5.12.1 Rabbit HRP-anti-human IgA

Polyclonal antisera to human IgA (α-chain specific) prepared in rabbits, was conjugated with horseradish peroxidase (Dako, Code number 216).

2.5.12.2 Sheep HRP-anti-human IgG

The IgG fraction of sheep anti-human IgG was conjugated with horseradish peroxidase was conjugated to HRP (SAPU, product number S 079-201).

2.5.12.3 Donkey HRP-anti-sheep/goat IgG

The IgG fraction of donkey anti-sheep/goat IgG was conjugated to horseradish peroxidase (SAPU, product code S048-201).

2.6 Single radial immunodiffusion plates

2.6.1 Nor-Partigen IgA

Immunodiffusion plates for determination of human IgA (Behring, product number OSLM 03) contained monospecific antiserum to human IgA (α chain specific) in agarose gel (assay range 0.42 - 6.34 g/l).

2.6.2 Nor-Partigen IgM

Immunodiffusion plates for determination of human IgM (Behring, product number OSLP 03) contained monospecific antiserum to human IgM (μ chain specific) in agarose gel (assay range 0.32 - 4.83 g/l).

2.6.3 Nor-Partigen IgG

Immunodiffusion plates for determination of human IgG (Behring, product number OSLN 03) contained monospecific antiserum to human IgG (γ chain specific) in agarose gel (assay range 0.23 - 3.4 g/l).

2.7 SDS-PAGE

Proteins were separated by SDS-PAGE using the SDS-discontinous system of Laemmli (1970) on a mini-protein II cell (Bio-Rad Laboratories Ltd., Watford, Hertfordshire, U.K). Equal volumes of protein sample and sample buffer were mixed and heated to 100 °C for 3 min. Approximately 20 µl of sample were applied to each lane and electrophoresis carried out at a constant voltage of 100 V through the stacking gel (10% acrylamide) and a constant voltage of 150V through the resolving gel (10% acrylamide). Proteins were visualised by staining with Coomassie blue [0.5% (w/v) Coomassie brilliant blue in 25% (v/v) propan-2-ol + 10% (v/v) glacial acetic acid] followed by destaining with 10% (v/v) glacial acetic acid. Molecular weight markers (Sigma) in the range of 26,600 to 180,000 were run in parallel. The meningococcal

outer membrane proteins were identified by their molecular weight as defined in Tsai *et al.*, 1981.

2.8 Flow cytometric technique

2.8.1 Monoclonal anti-human IgG

Monoclonal anti-human IgG (Fc specific) is of the mouse IgG2a subclass as determined by double diffusion assay, clone HP-6017 (Sigma, Pool, Dorset, product number 1 6760).

2.8.2 Pool

Two pools of saliva, one from secretors (n=120) and one from non-secretors (n=120), were prepared from material collected in the survey of school children. Each contained a total of 120 ml.

2.8.3 Coupling protein to Sephasose 4B

Monoclonal anti-human IgA, monoclonal anti-human IgG and monoclonal anti-human IgM were used for the purification of IgA, IgM and IgG from saliva. The three monoclonal antibodies were coupled to cyanogen bromide activated Sepharose 4B beads (Pharmacia, Uppsala, Sweden) (0.5 g of dry beads can bind up to 3.5 mg of protein). Impurities were washed away from the beads by mixing them with 10 ml of mM HCl (pH 3.0) for 15 min at room temperature. The beads were pelleted by centrifugation at 50 g for 5 min. Each of the three antibodies were diluted in coupling buffer (0.1 M sodium bicarbonate + 0.5 M sodium chloride, pH 9.0).

The gel-protein mixtures were rotated overnight at 4 °C. Unbound material was removed by centrifugation at 50 g for 5 min. The beads were washed three times with 15 ml NET buffer (0.15 M sodium chloride + 0.04 M Tris + 0.2 mM phenylmethyl sulfonyl fluoride, pH 7.0). Free active groups on the gel were blocked by mixing with

1 M ethanolamine (pH 8.0) for 2 h. Non-covently bound material was removed by three cycles of washing with 15 ml 0.1 M acetate buffer (0.1 M sodium acetate + 1 M sodium chloride, pH 4.0) and then with 15 ml 0.1 M bicarbonate (0.1 M sodium hydrogen carbonate + 1 M sodium chloride pH 8.0).

2.8.4 Antibody purification on Sepharose beads

The pool (5 ml) was mixed with 1.5 ml of anti-human IgA, anti-human IgG or anti-human IgM Sepharose 4B beads (see section 2.8.2). After overnight incubation at 4 °C the supernatant was removed and the Sepharose beads washed three times with 15 ml NET buffer. The antibodies were eluted by incubation with 3 ml of 1 M acetic acid for 30 min at 20 °C with continuous rotation. The elute was dialysed against three changes of PBS. The presence and purity of IgA, IgG and IgM antibodies was tested using ELISA system as described in sections 4.2.1.1 and 3.3.9.1 respectively.

The unbound material of the three pools was kept at -20 °C for further studies.

2.8.5 Analysis of cells by flow cytometery

Analysis was done on an EPICS "C" flow cytometer (Coulter Electronic, Luton, UK) equipped with a 5 watt laser using a power output of 300 mw at 488 nm. The cells were selected from a display of forward angle light scatter versus 90° light scatter by means of a bit map. More than 3,000 cells were analysed from each sample. The percentage of cells showing fluorescence greater than the background level was recorded on a one parameter histogram measuring fluorescence on a logarithmic scale. The mean fluorescence channel values for the positive cells were acquired from a one parameter histogram measuring fluorescence on a linear scale. The results were analysed by the immunoanalysis program (Coulter).

2.9 Solutions for bactericidal assay

2.9.1 Dulbecco's phosphate buffered saline

Dulbecco's phosphate buffered saline (D.PBS) (pH 7.1) was prepared from Dulbecco's A tablets (Oxoid Ltd., England) to give a solution containing sodium chloride (5 g/l). potassium chloride (0.2 g/l), sodium phosphate monobasic (1.15 g/l) and potassium phosphate dibasic (0.2 g/l). Phenol red indicator was added to give a final concentration of 0.0002%.

Every 100 ml D.PBS was supplemented with 0.5 ml Dulbecco's B containing calcium chloride (20 g/l) and magnesium chloride (20 g/l) (D.PBS + B).

2.9.2 GC Broth

GC Broth (Difco) (pH 7.2) was prepared to give a solution containing protease peptone no. 3 (15 g/l), corn starch (1 g/l), potassium phosphate dibasic (4 g/l), potassium phosphate monobasic (1 g/l), and sodium chloride (5 g/l).

2.9.3 Sheep red blood cells

Whole blood taken aseptically from healthy sheep was supplied as a 50% (v/v) mixture in Alsever's solution (SAPU, product number S 033-220).

2.9.4 Donkey anti-sheep/goat IgG

Anti-sheep/goat IgG is a pool of selected antisera obtained from donkeys immunized with sheep/goat IgG (SAPU, product number S 024-220).

Development of ELISA for detection of total and specific antibodies to *Neisseria*

3.1 Introduction

Since the introduction of enzymes as markers for the labelling of antigens and antibodies (Nakane and Pierce, 1966), immunoenzymatic techniques have been developed and diversified for the qualitative and quantitative detection of macromolecules. These methods are routinely used for the localisation of antigens (or antibodies) on tissues, for the detection of antigens (or antibodies) immobilised on various solid phases, for titration of antibodies and the precise measurement of antigens and antibodies.

Antibodies to whole bacteria have been detected by fluorescent antibody tests (Lehner *et al.*, 1976) or by agglutination (Challacombe and Lehner, 1976). These assays are not very sensitive and are not strictly quantitative. Detection of specific antibodies by the ELISA method was first described by Engvall and Perlmann (1972). The use of solid phase immunoassays offers an attractive approach since they combine sensitivity and specificity and can detect specific isotypes. In addition, the use of ELISA microtitre plate readers enables a large number of reactions to be read in a short time.

The growing interest in development of meningococcal vaccines has prompted the establishment of ELISA methods for assay of responses to the vaccines (Rosenqvist *et al.*, 1988; 1991). The purpose of this study was the development of ELISA methods capable of reliably quantifying total and specific serum and salivary antibodies. To optimise the techniques, the following parameters were analyzed: time and temperature of incubation; buffers used for washing; type and concentration of the reagents; concentration of saliva and serum in the assay. Information from these analyses was

used to choose conditions under which the background absorbance was reduced by preventing non-specific interactions, but at the same time, maximum immunological and enzymatic reactions were obtained.

3.2 Materials and methods

3.2.1 ELISA system

3.2.1.1 General conditions of the assay

3.2.1.1.1 Detection of the IgA and IgM

For the measurement of total IgM and IgA, a capture ELISA system was used. The wells of polystyrene microtitre plates were coated overnight at 4 °C with either mouse monoclonal anti-human IgM or mouse monoclonal anti-human IgA diluted in coating buffer. The coated plates were washed and blocked with blocking buffer. Serum or saliva specimen were added and incubated at room temperature. Plates for detecting IgM were washed and sheep anti-human IgM was added. After washing, HRP labelled donkey anti-sheep/goat IgG was added to the plates. For IgA plates, HRP labelled rabbit anti-human IgA was added to the plates.

Finally, the plates were washed and substrate was added. The colour change was stopped after 20-30 min. by adding 12.5% H_2SO_4 (Figures 3.1 and 3.2).

3.2.1.1.2 Detection of total IgG

To determine total IgG, serum or saliva specimens diluted in coating buffer were added directly to ELISA plates and incubated overnight. After blocking with blocking buffer HRP labelled sheep anti-human IgG was added to the plate. The plates were then treated as for IgA and IgM (Figure 3.3).

Optical density (OD) was determined by an ELISA plate reader (Dynatech). Readings for the test samples were corrected by subtracting the OD of the corresponding blank which contained no serum or saliva; the OD of the blank was usually between 0.056 and 0.067. Results obtained with a series of twofold dilutions of standard human serum containing known concentrations of immunoglobulin (mg/ml) were used to construct a graph from which the OD readings of the specimens could be used to calculate the immunoglobulin levels.

For detection of specific antibodies, plates were coated with *N. meningitidis* or *N. lactamica* isolates. Serum or saliva was added to the wells. The assay was carried out as described above to detect antibodies of each isotype (Figure 3.4).

The above variables were each defined by experiments outlined below.

3.2.1.2 Coating with whole bacteria

Bacterial suspensions prepared as described in (section 2.4.5) were prepared in bicarbonate buffer or PBS. The bacteria (100 µl) were added to wells of microtitre plates and the plates incubated overnight at 4 °C.

3.2.1.3 Determination of the dilutions of serum, saliva and reagents

For detection of total IgA and IgM, different concentrations of capture antibody (anti-human IgA and anti-human IgM) were used to coat the plates with either bicarbonate buffer or PBS. Serum or saliva specimens and standard serum (50 µl) were serially diluted in blocking buffer and added to the capture antibody. HRP-conjugate antibodies were also tested in the same manner. Assays with different dilutions were performed to establish: (a) optimal conditions for coating the plate; (b) concentrations of all the reagents; (c) the OD obtained with the dilutions of serum and saliva fitted within the curve obtained with standard serum.

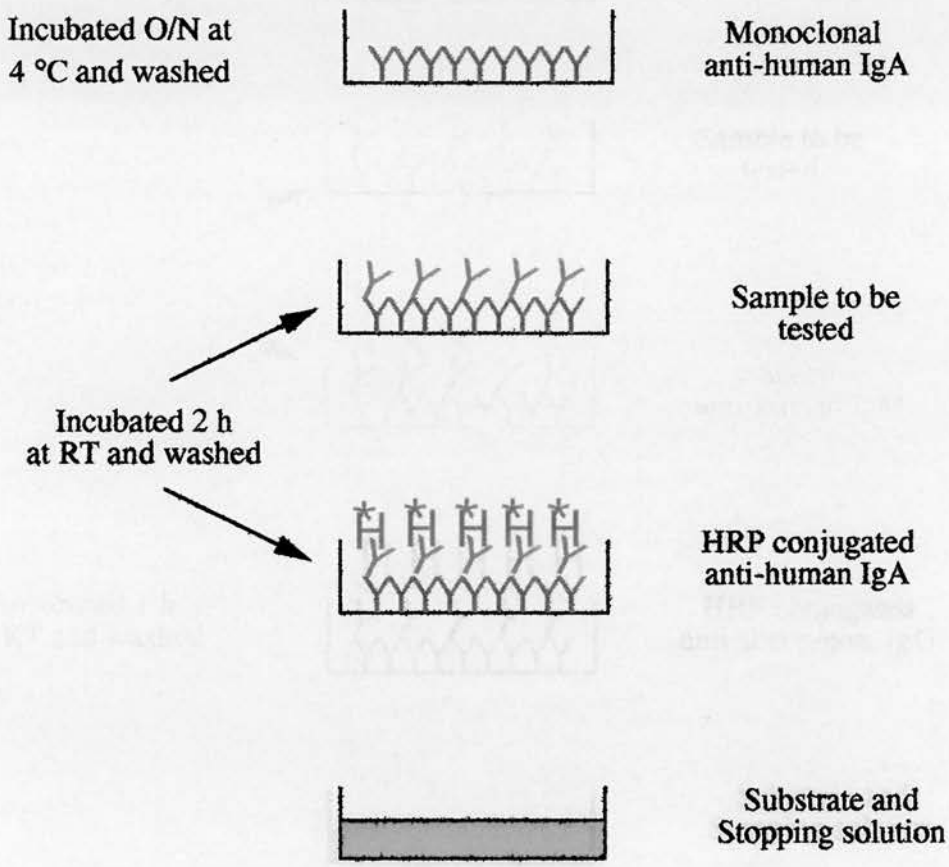

Figure 3.1: Capture ELISA for detection of total IgA in serum and saliva

O/N = Overnight RT = Room temperature

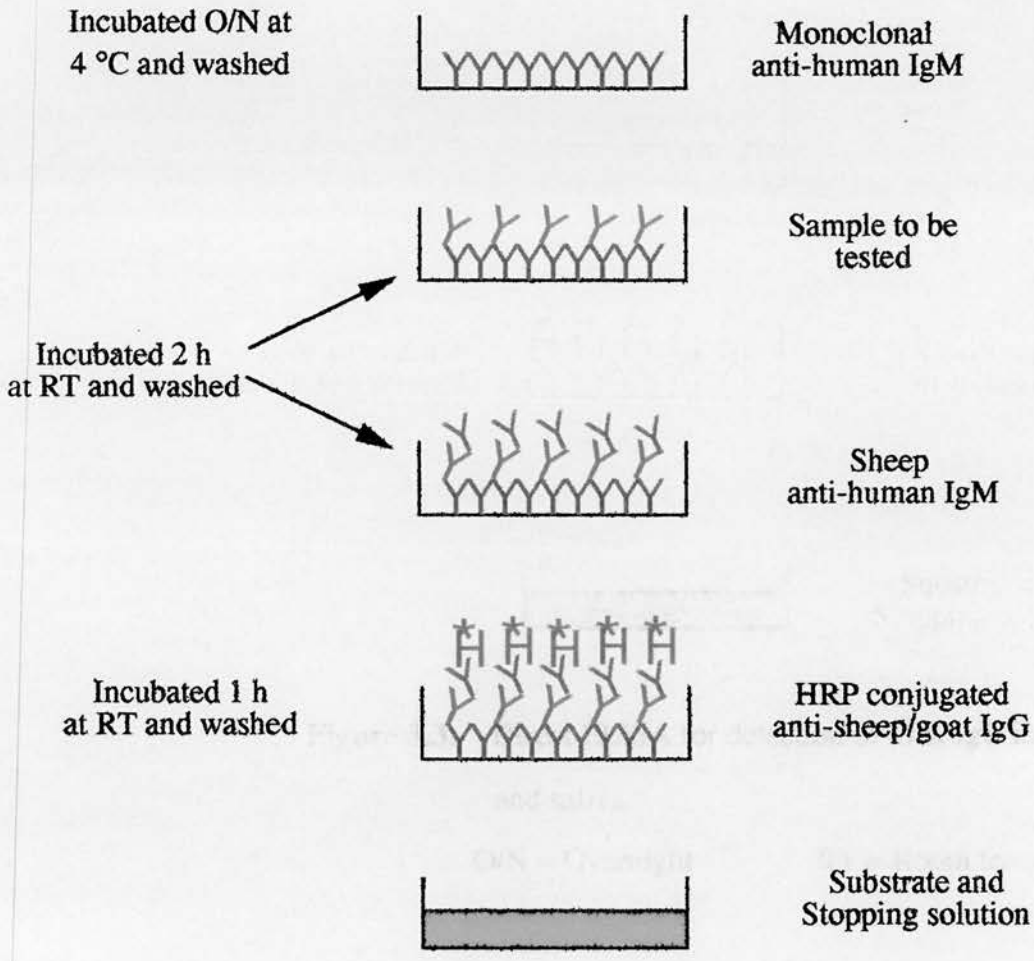

Figure 3.2: Capture ELISA for detection of total IgM in serum and saliva

O/N = Overnight RT = Room temperature

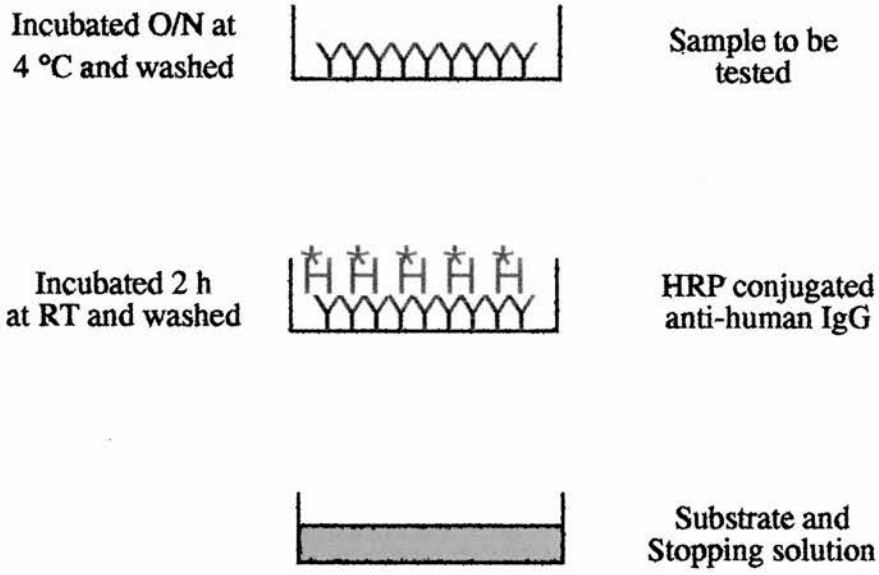

Figure 3.3: Direct ELISA for detection of total IgG in serum and saliva

O/N = Overnight RT = Room temperature

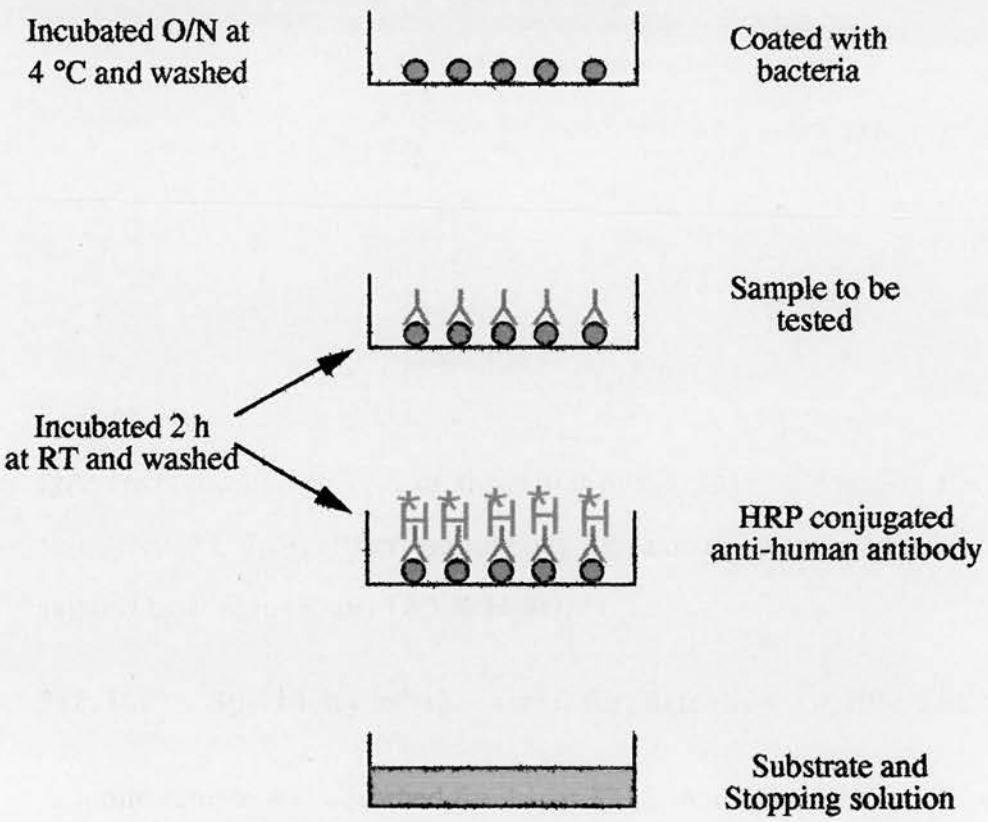

Figure 3.4: ELISA for detection of anti-meningococcal antibodies in serum and saliva

O/N = Overnight RT = Room temperature

For determination of total IgG, various dilutions of serum, saliva or standard serum diluted in coating buffer (100 µl) were added directly to the plates.

3.2.1.4 Time Course

Serum, saliva and HRP conjugated antibodies (HRP anti-human IgA, HRP anti-human IgG, sheep anti-human IgM and HRP anti-sheep/goat IgG) were tested at different times (0 - 4 h) to determine the optimum incubation period for detection of serum and salivary antibodies.

3.2.1.5 Specificity of the binding assay for detection of total antibodies

To illustrate that the ELISA is specific, the following method was devised. Mouse monoclonal anti-human IgA or anti-human IgM were added to separate plates (100 µl) then a constant dilution of serum was added (50 µl). To separate wells in each plate one of the three labelled antibodies (50 µl) was added: HRP conjugated anti-human IgA; HRP anti-human IgG; or sheep anti-human IgM followed by HRP conjugated anti-sheep/goat IgG. After washing, the substrate (50 µl) was added and the reaction stopped by adding (50 µl) 12.5 % H_2SO_4.

3.2.1.6 Specificity of the assay for detection specific antibodies

A serum sample was adsorbed for 1 h at 37 °C then overnight at 4 °C with different strains of *Neisseria;*: B:4:P1.15; NG: 4: -; *N.gonorrhoeae* ; and *N. lactamica*.. In addition, serum was incubated with purified outer membrane proteins (OMPs) from the outbreak strain (section 2.7). OMPs were added to the serum and incubated for 1 h at 37 °C followed by overnight incubation at 4 °C. Binding of unabsorbed and adsorbed sera to the outbreak strain was examined by ELISA.

3.2.1.6.1 Preparation of OMPs

Outbreak strain B:4:P1.15 was grown on MNYC under standard conditions. Bacteria were harvested into 60 ml of PBS (pH 7.2) to give a heavy suspension and centrifuged at 2000 g for 20 min. The bacteria were resuspended in 30 ml of PBS and sonicated on ice three times, 6 ml at a time at an amplitude of 6 microns for 60 sec (MSE, Leicestershire, UK). Unbroken cells were removed by centrifugation at 3000 g for 20 min at 4 °C; and, the total cell envelope fraction from the supernatant was pelleted by centrifugation at 60,000 g at 4 °C for 60 min. The pellet was resuspended in 30 ml of 2 % (w/v) sodium N-lauroylsarcosine ('Sarkosyl', Sigma) for 20 min at room temperature. This detergent selectively solubilizes the inner membrane from the crude envelope preparations and provides a method for obtaining the outer membrane (Hancock and Poxton, 1988). The remaining outer membrane fraction was pelleted by centrifugation at 60,000 g at 4 °C for 60 min and resuspended in distilled waster.

3.2.2 Single radial immunodifusion (SRID) system

Immunoglobulins were quantitated by the SRID technique of Mancini *et al.*, (1965) with commercially available antibody agar plates (IgA, IgG and IgM) (section 2.6). To check the accuracy of the system, a control serum was added to one well of each plate. Wells two to twelve were used for the specimens to be examined. Serum (5 µl) was added to each well with a Behring dispenser. Serum diluted in isotonic saline was used for IgA, IgG and IgM. The plates were incubated for 48 h in a moist box at 4 °C. Diameters of precipitin rings were measured in four different directions in millimeters and the average was taken. All samples with diffusion rings larger than 9.3 mm were retested with more dilute serum. SRID readings were converted into g/l value by the calibration table provided by the manufacture.

3.2.3 Comparison between ELISA and SRID

For comparison between ELISA and SRID methods, 16 serum samples were used to compare the values obtained by the ELISA with those obtained by commercially available SRID.

3.3 Results

Four serum and saliva samples were chosen randomly from the collection to determine the optimum dilution of serum, saliva and working reagents.

3.3.1 Determination of optimal concentration of antibodies bound to the solid phase

To obtain the highest level of sensitivity of the ELISA, it was necessary to determine the optimum concentration of capture antibody to provide an acceptable level of capture antibody to bind the IgA or IgM from the samples and to prevent non-specific uptake of immunoglobulins from the serum or saliva. Serum or saliva specimens undiluted were incubated in wells pre-incubated with 100 µl of different dilutions of capture antibody in coating buffer. Optimum readings were observed at dilutions from 1/400 to 1/1000 for both mouse monoclonal anti-human IgA and mouse monoclonal anti-human IgM (Figure 3.5). At higher concentrations, the absorbance reached a plateau. Both monoclonal antibody preparations gave similar curves. A dilution of 1/500 for both monoclonal antibodies were used in subsequent experiments.

For IgG assays, the standard and test sera diluted in coating buffer were applied directly onto the plates; the graph was linear between 1/51200 to 1/409600 (Figure 3.6).

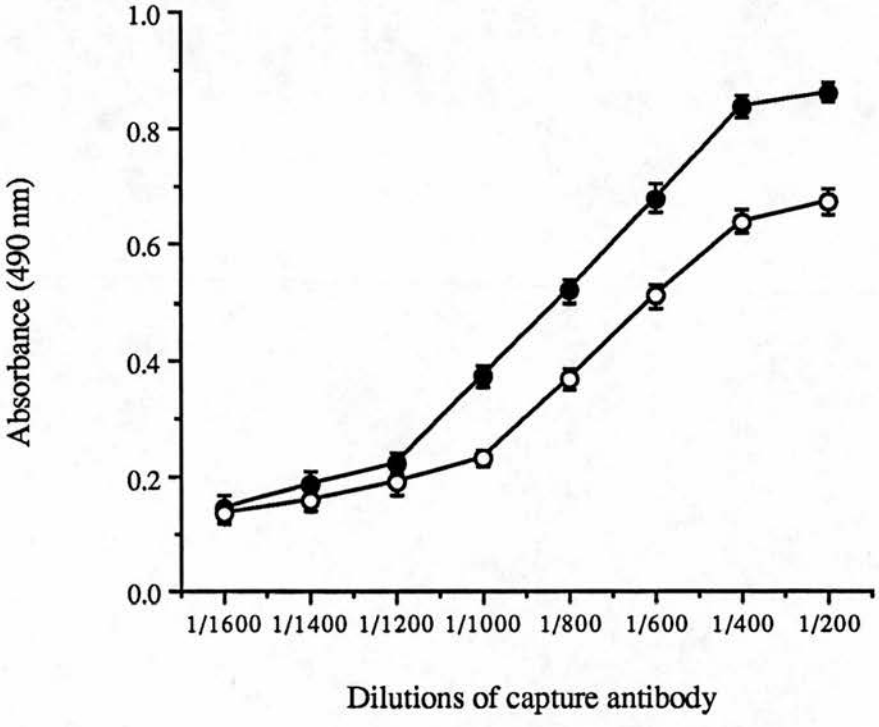

Figure 3.5: Titration of monoclonal anti-human IgA (closed) and monoclonal anti-human IgM (open) for capture ELISA with standard serum. Each point represents the mean ± SEM of 4 replicates.

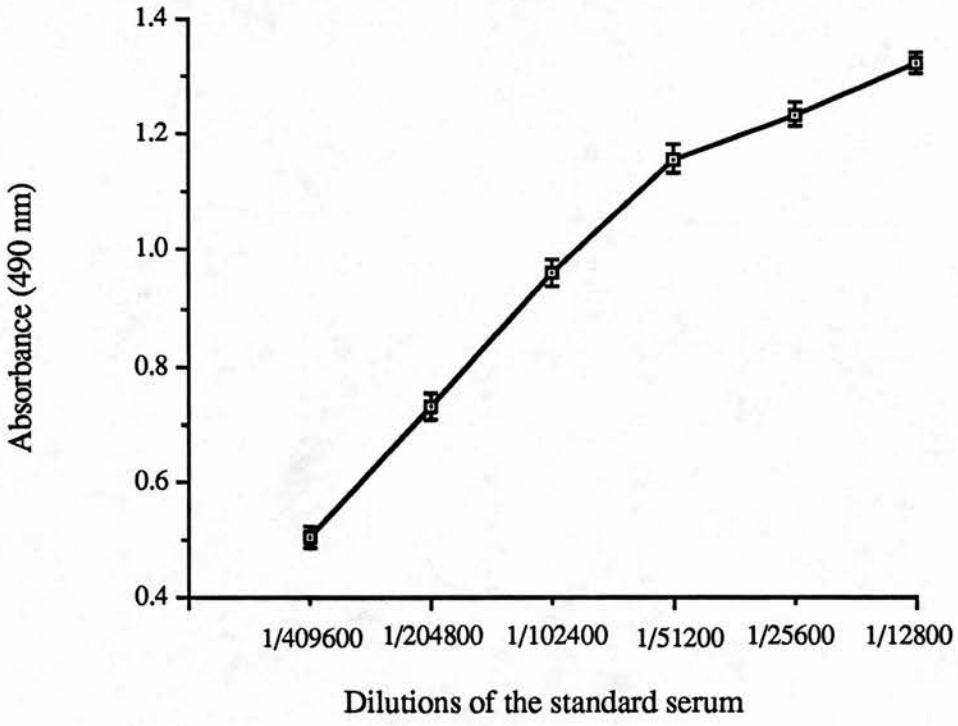

Figure 3.6: Titration of IgG in the standard serum coated directly onto ELISA plates. Each point represents the mean ± SEM of 8 replicates.

3.2.2 Adsorption of bacteria onto the solid phase

Binding of the bacteria (outbreak strain B:4:P1.15) to the microtitre plates was detected with antibody from a single donor. Formaldehyde treated bacterial preparations 4.8 x 10^8/ml could not be bound to the solid phase if suspended in PBS. High levels of attachment were achieved when carbonate-bicarbonate buffer was used to suspend the same concentration of bacteria for detection of IgA, IgM and IgG (Figures 3.7 - 3.9). The results of titrations indicated that at concentrations between 2.4 x 10^8 and 4.8 x 10^8 high OD values were obtained within a reasonable reaction time (20 min) with the substrate. Similar results were found for all strains examined.

3.3.3 Determination of the optimal dilutions of serum and saliva

Serial dilutions of serum or saliva were prepared from one donor and assayed for total levels of IgA, IgG and IgM. A dilutions of 1/2000 of serum and 1/10 of saliva were found suitable for total IgA and IgM (Figures 3.10 and 3.11). For detection of IgG in serum a dilution between 1/30000 and 1/50000 (Figure 3.12) and 1/2 of saliva in coating buffer was used.

For measurement of specific antibodies to different isolates, undiluted serum or saliva was used for IgA, IgG and IgM after coating the plate with bacteria (Figure 3.13).

All the above dilutions were selected to obtain the highest level of sensitivity and fitted within the range of of the slope standard.

3.3.4 Determination of the optimal concentration of HRP-labelled antibodies

Four randomly chosen serum or saliva specimens were used in these assays.

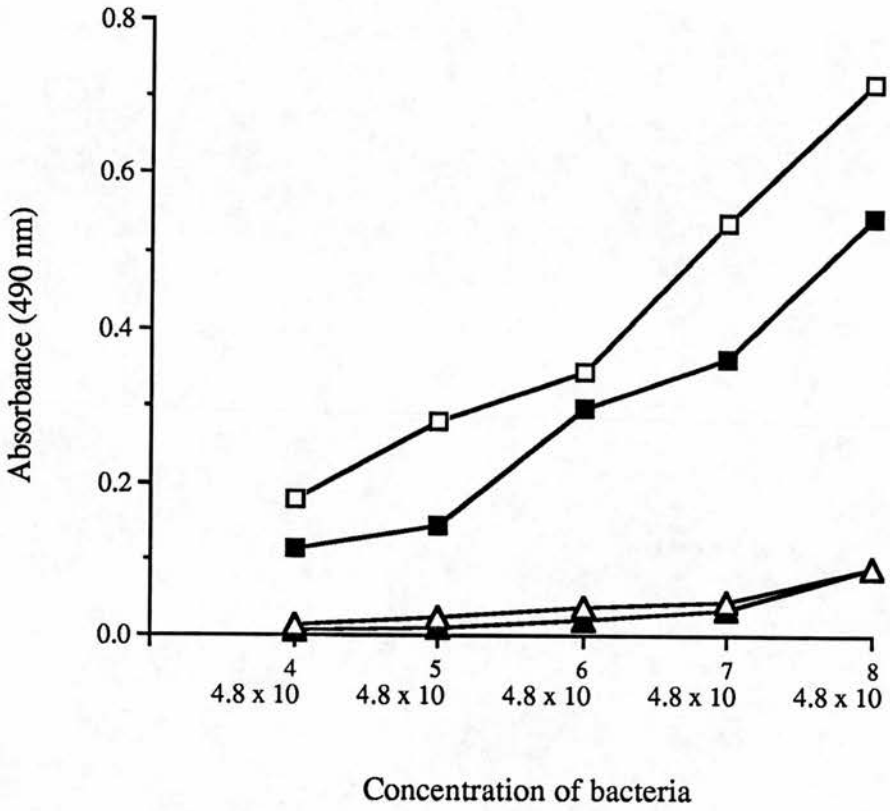

Figure 3.7: Binding of bacteria to ELISA plates with bicarbonate buffer (■, □) or PBS (▲, △) for detection of serum (closed) and salivary (open) IgA

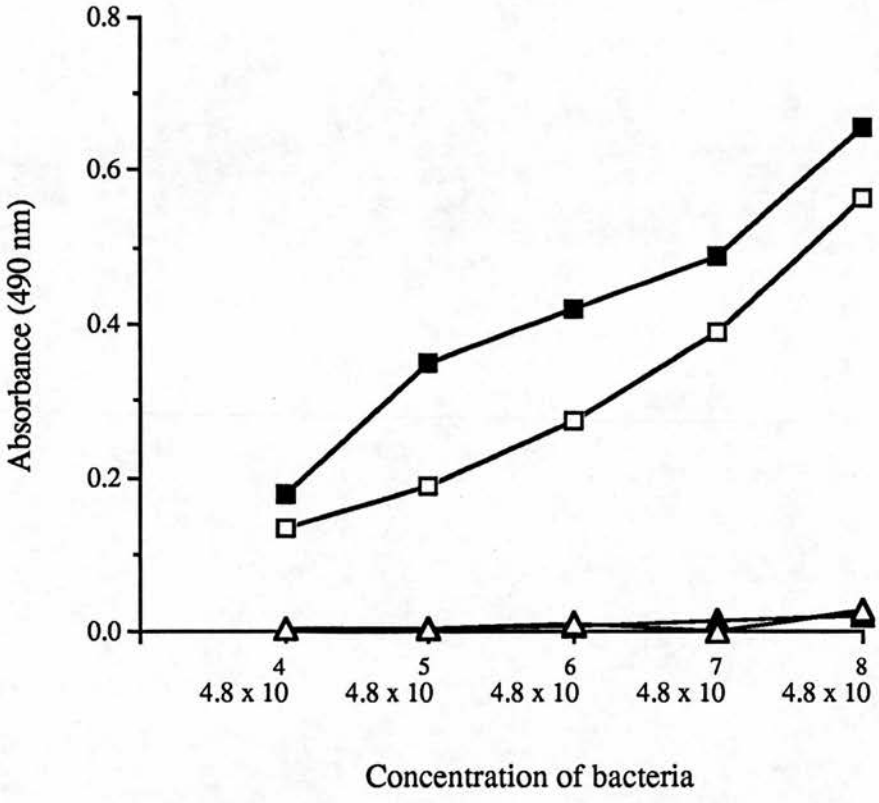

Figure 3.8: Binding of bacteria to ELISA plates with bicarbonate buffer (■, □) or PBS (▲, △) for detection of serum (closed) and salivary (open) IgM

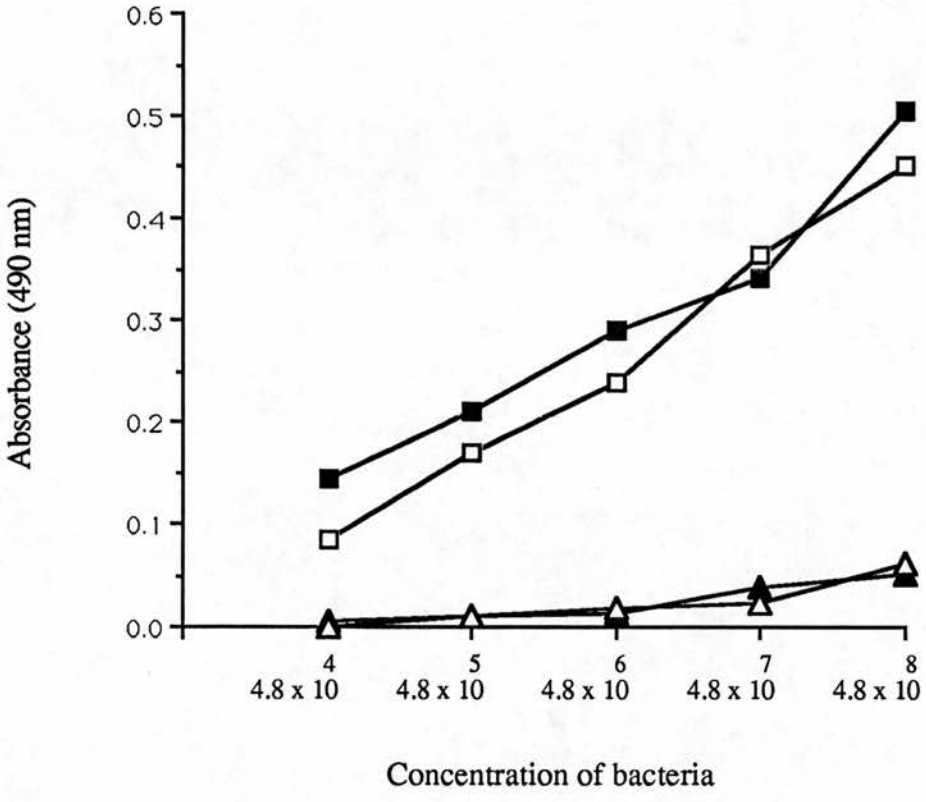

Figure 3.9: Binding of bacteria to ELISA plates with bicarbonate buffer (■, □) and PBS (▲, △) for detection of serum (closed) and salivary (open) IgG.

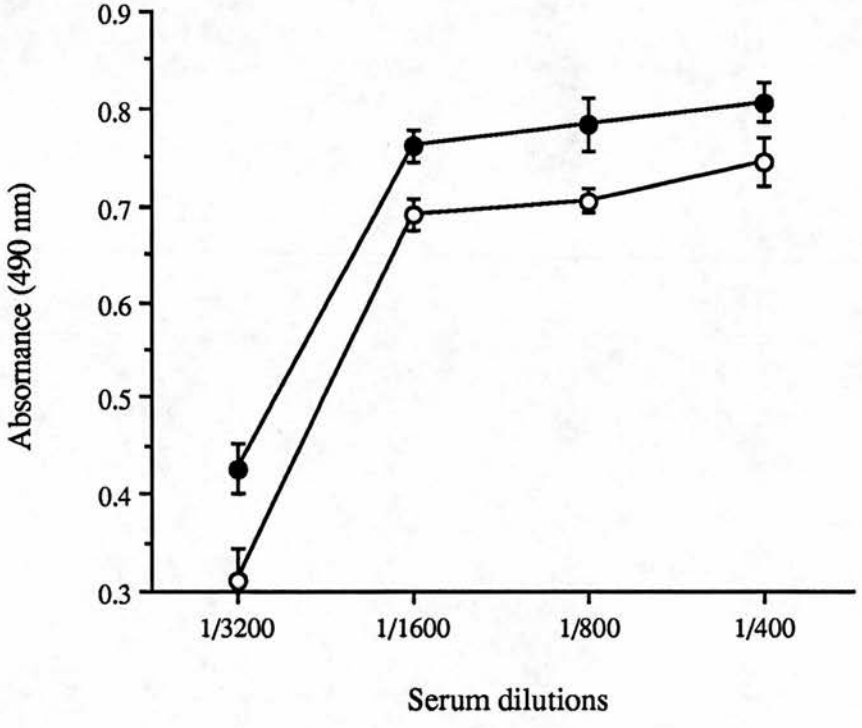

Figure 3.10: Titration of serum for detection of total IgA (open) and IgM (closed). Each point represents the mean ± SEM optical density obtained from 4 replicates.

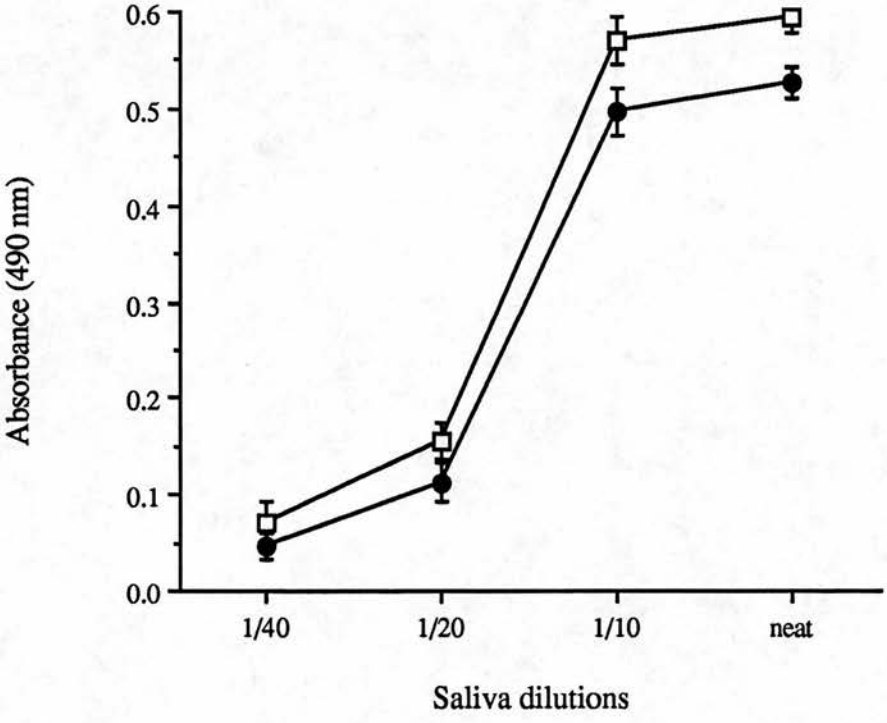

Figure 3.11: Titration of saliva for detection of total IgA (open) and IgM (closed). Each point represents the mean ± SEM optical density obtained from 4 replicates.

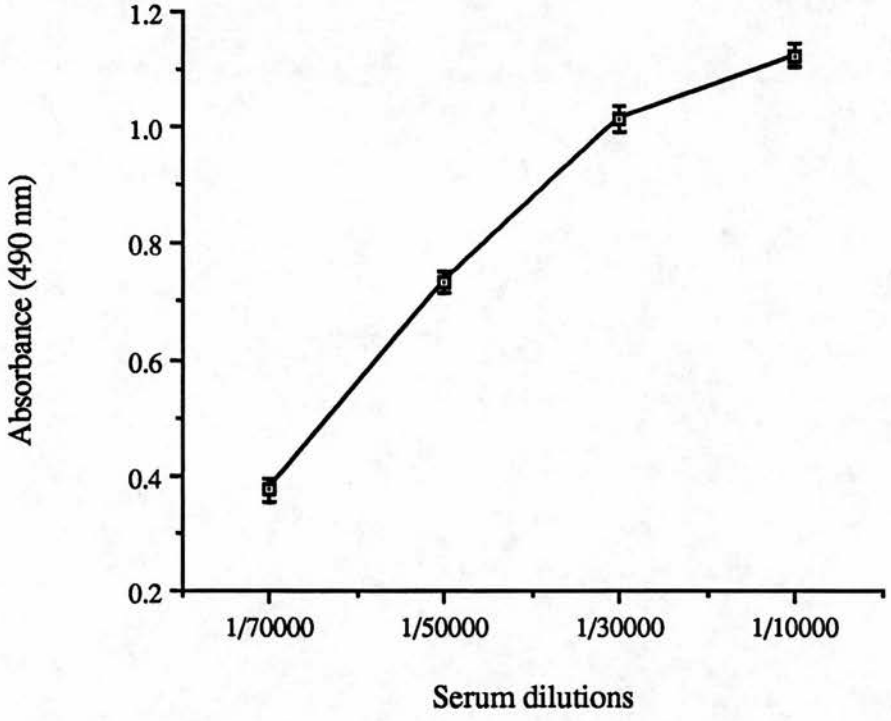

Figure 3.12: Titration of serum for detection of total IgG. Each point represents the mean optical density ± SEM obtained from 4 replicates.

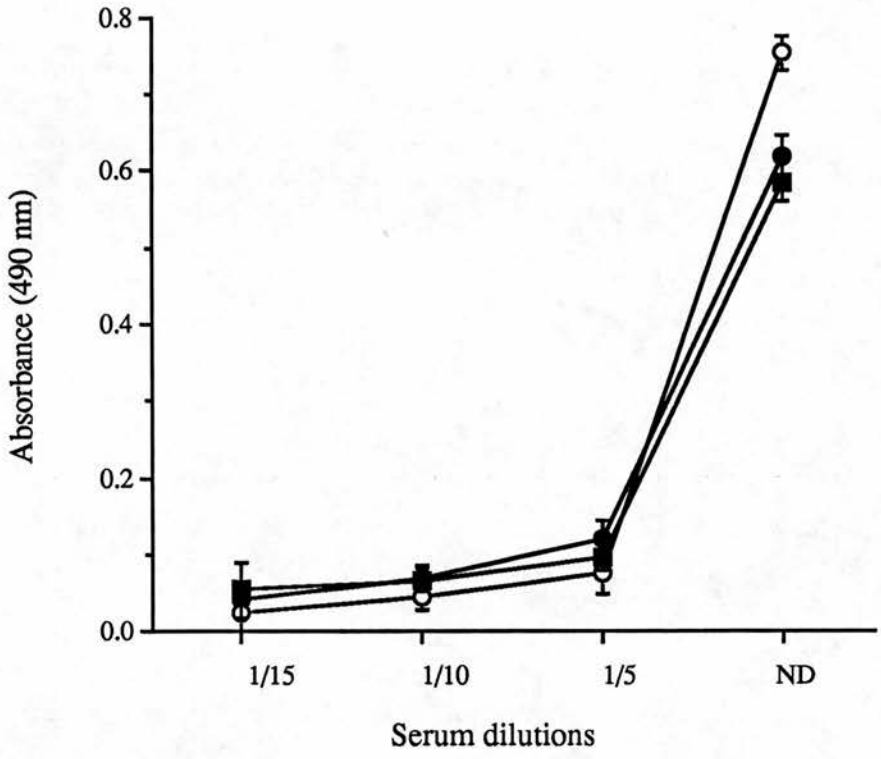

Figure 3.13: Titration of serum IgA (O), IgG (●) and IgM (■) to meningococci. Each point represents the mean ± SEM optical density obtained from 4 experiments

(ND = non-diluted)

3.3.4.1 Detection of IgA and IgG

The dilutions of HRP-labelled antibodies used to detect IgA or IgG bound to the solid phase are shown in (Figures 3.14 and 3.15). They were tested at dilutions from 1/100 to 1/1600 for HRP anti-human IgA and 1/10 to 1/120 for HRP anti-human IgG in doubling dilution steps. With increasing amounts of HRP anti-human IgA or HRP anti-human IgG, the enzymatic activity increased. A dilutions of 1/500 of HRP-anti-human IgA and 1/20 of HRP-anti-human IgG were used in subsequent assays.

3.3.4.2 Detection of IgM

For detection of IgM, sheep anti-human IgM was added first followed by HRP anti-sheep/goat IgG. Anti-human IgM was serially diluted from 1/40 to 1/200, and HRP sheep/goat IgG was diluted from 1/10 to 1/100 (Figures 3.16 and 3.17). Dilutions of the labelled antibody resulted in dose response curves in assays for detection of total or specific antibodies in serum or saliva. On the basis of these results, further tests were routinely performed with dilutions of 1/80 and 1/20 for anti-human IgM and HRP sheep/goat IgG respectively.

3.3.4.3 HRP-conjugated reagents

Each lot of HRP-conjugated reagent was checked for non-specific binding to the coating and the blocking reagent (section 2.5.6). Any conjugates yielding a non-specific reaction with either the coating antigen or bovine serum albumin were rejected.

3.3.5 The effect of incubation time

Incubation times for the various stages of the assay were selected to combine high sensitivity with timing convenient for the working day. Figure 3.18 demonstrates the time course for binding of IgA from serum or saliva to the capture antibody; a levelling off was observed was reached between 2 and 4 h at room temperature. Similar results

were obtained in other assays for detection of total IgM or IgG and for detection of these isotypes specific for the bacteria in serum and salivary antibodies (data not shown). The influence of incubation time of labelled antibody (HRP anti-human IgA, HRP anti-human IgG) with the solid phase was determined between 0 - 4 h (Figure 3.19). Anti-human IgM and HRP anti-sheep goat IgG were tested within the same range (Figure 3.20). On the basis of these results, the tests were routinely performed for 2 h for all the incubation periods except for donkey HRP anti-sheep/goat IgG which was 1 h for both serum and saliva, total and specific. For the incubation with substrate a maximum of 20 min. at room temperature was selected. Within this period, the reaction was stopped when strong colour had developed in the positive control and the negative control was still colourless.

3.3.6 Serum standard

Using a standard serum, calibration curves for IgA, IgM and IgG were generated. A linear relationship was obtained for the three classes of antibodies within the dilutions of the standard that were used (Figures 3.21 - 3.23).

3.3.7 Linearity

Linearity was tested on the same plate with serial dilutions of three samples selected randomly and a pool of twenty sera chosen randomly. Figures 3.24 - 3.26 demonstrate the linearity of the four samples tested over a range of dilutions. For each isotype, the linear part of the graph for test sera was within the linear portion of their corresponding standard curve. The results of the linearity are also presented as coefficient of variance which ranged from 1.5 - 5.1% for the three immunoglobulin isotypes (Table 3.1).

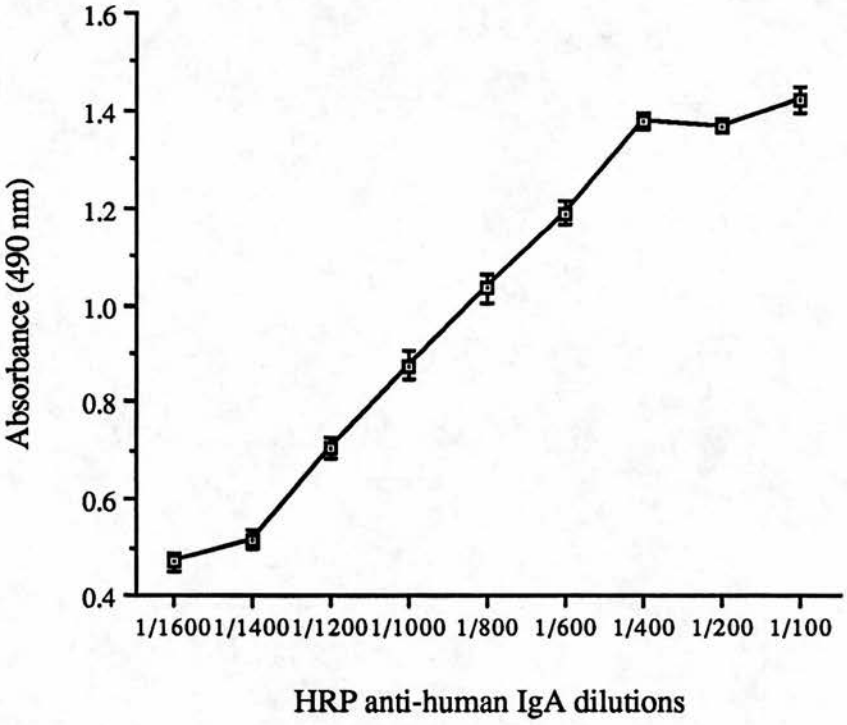

Figure 3.14: Titration of HRP anti-human IgA. Each point represents the mean ± SEM of 4 replicates.

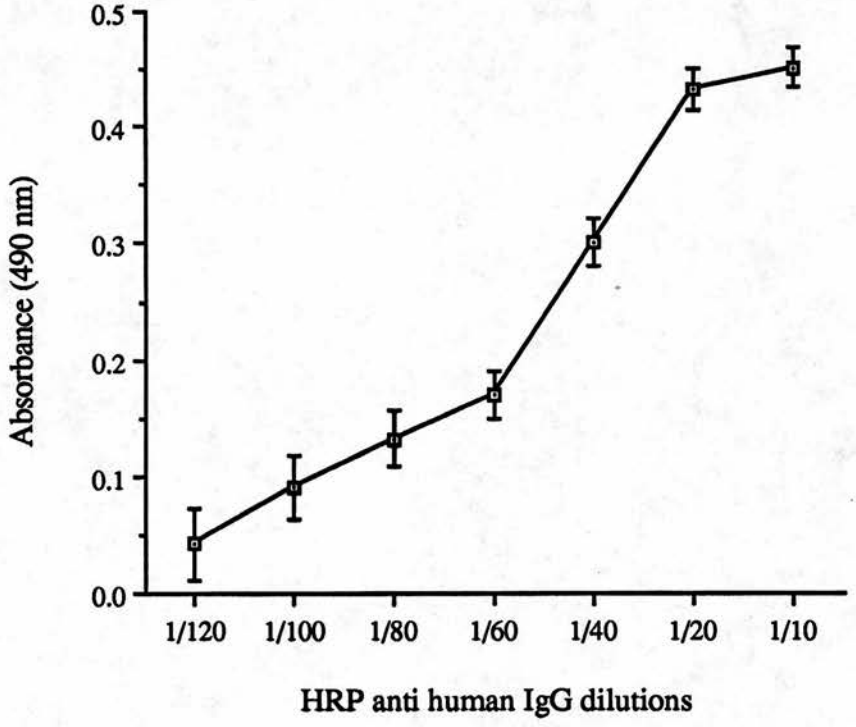

Figure 3.15: Titration of HRP anti-human IgG. Each point represents the mean ± SEM of 4 replicates.

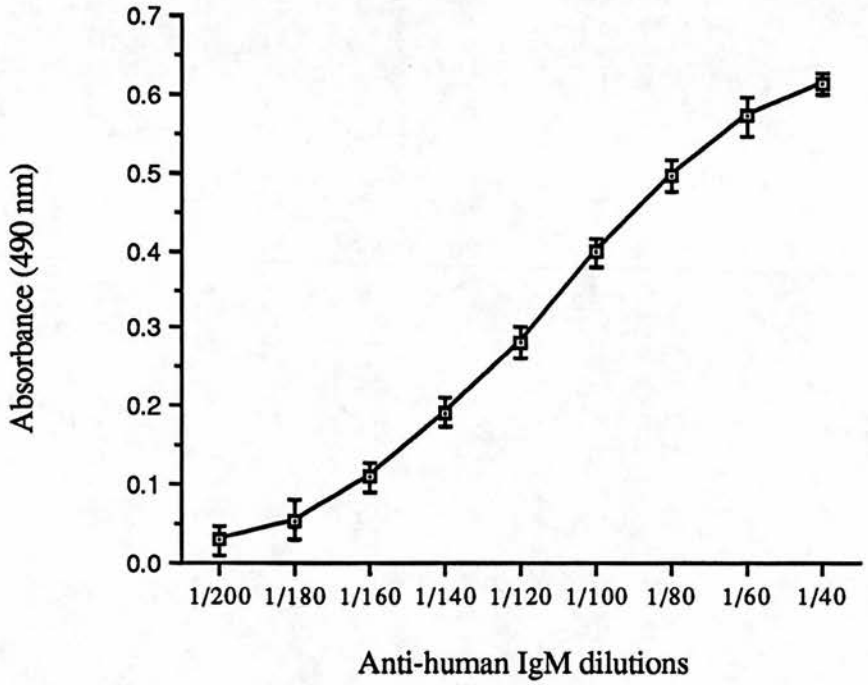

Figure 3.16: Titration of sheep anti-human IgM with HRP anti-sheep/goat IgG (1/20). Each point represents the mean ± SEM of 4 replicates.

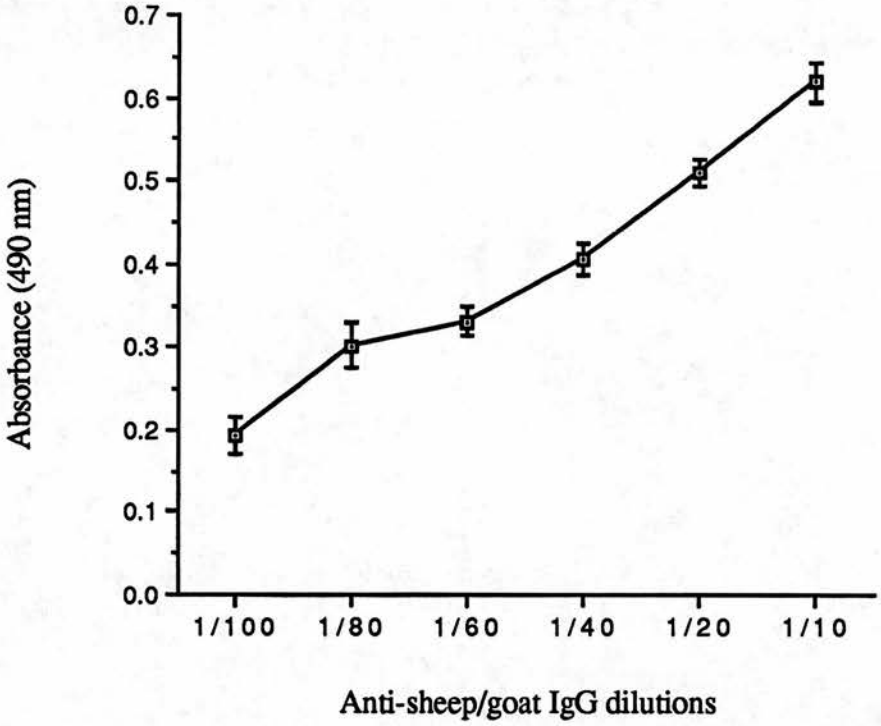

Figure 3.17: Titration of HRP anti-sheep/goat IgG with sheep anti-human IgM. Each point represents the mean ± SEM of 4 replicates.

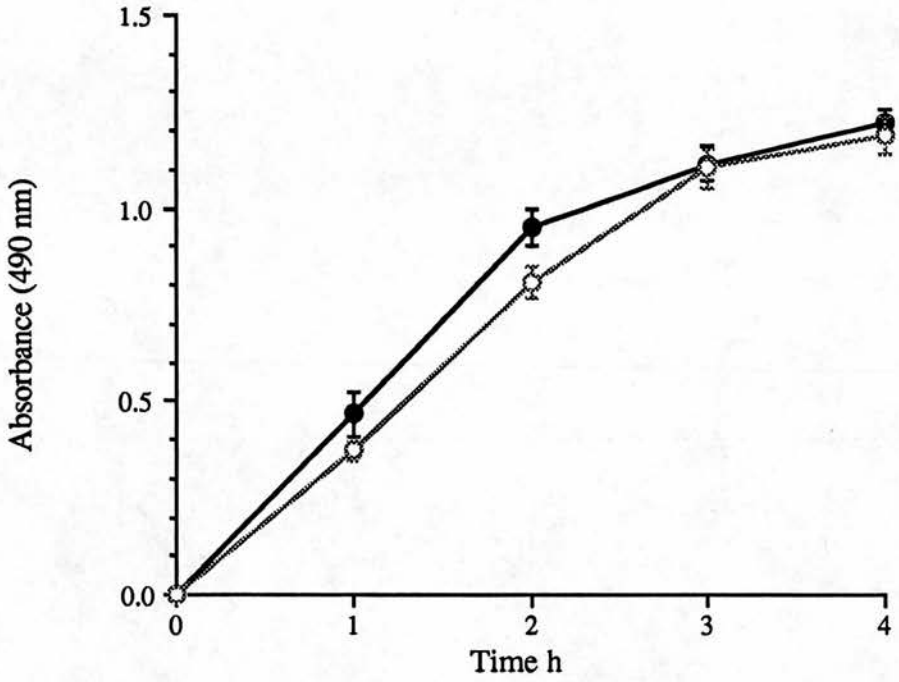

Figure 3.18: The time course for binding of total IgA from serum (●) or saliva (○) to capture antibody. Values are means ± SEM of duplicate wells from a representative experiment.

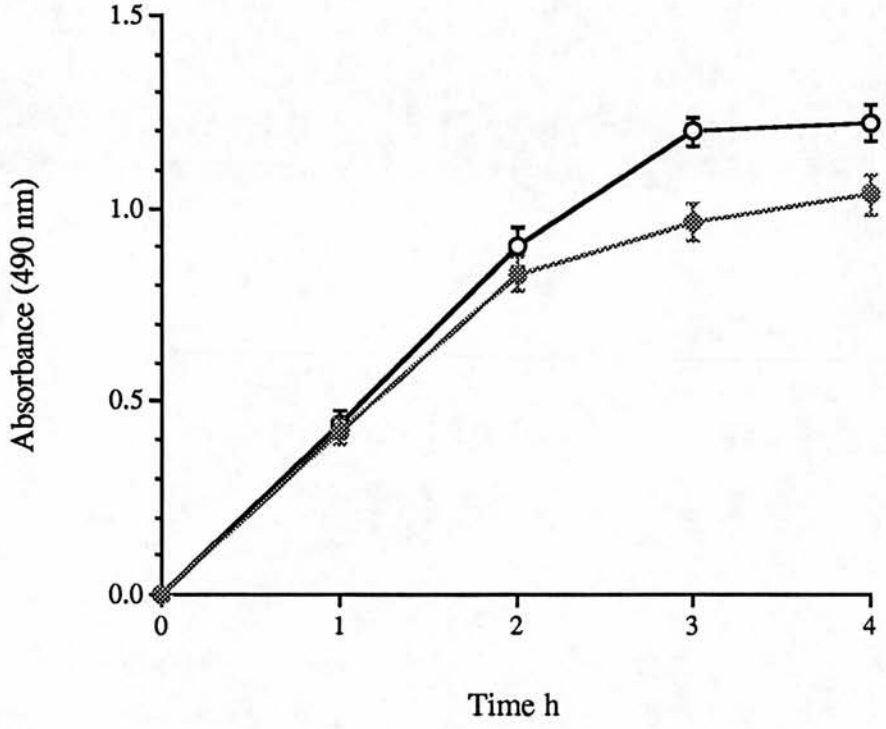

Figure 3.19: Binding of of HRP anti-human IgA (○) and HRP anti-human IgG (●) to serum IgA and IgG respectively as a function of time. Values are means ± SEM of duplicate wells from a representative experiment.

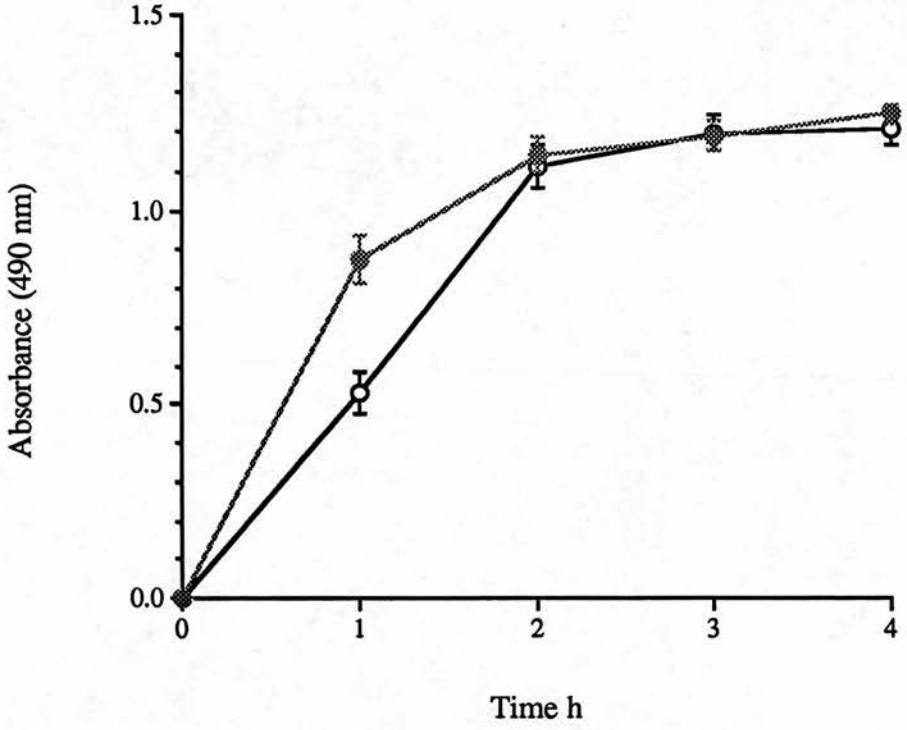

Figure 3.20: Binding of anti-human IgM (o) and HRP anti-sheep/goat IgG (●) to serum IgM as a function of time. Values are means ± SEM of duplicate wells from a representative experiment.

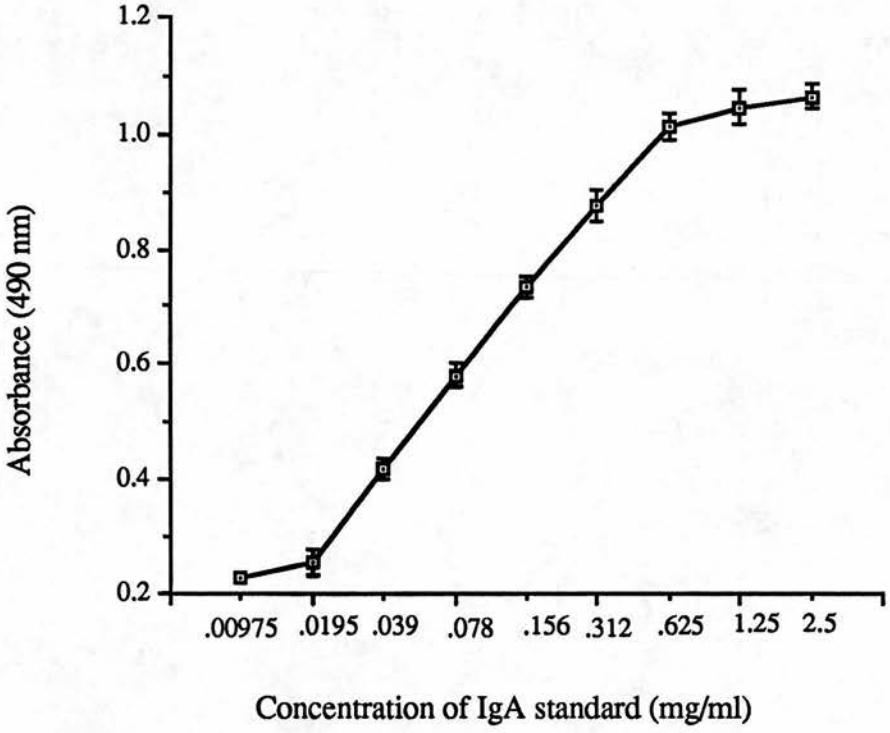

Figure 3.21: Optical density of ELISA for detection of IgA in the serum standard. Each point represents a mean ± SEM of 5 replicates.

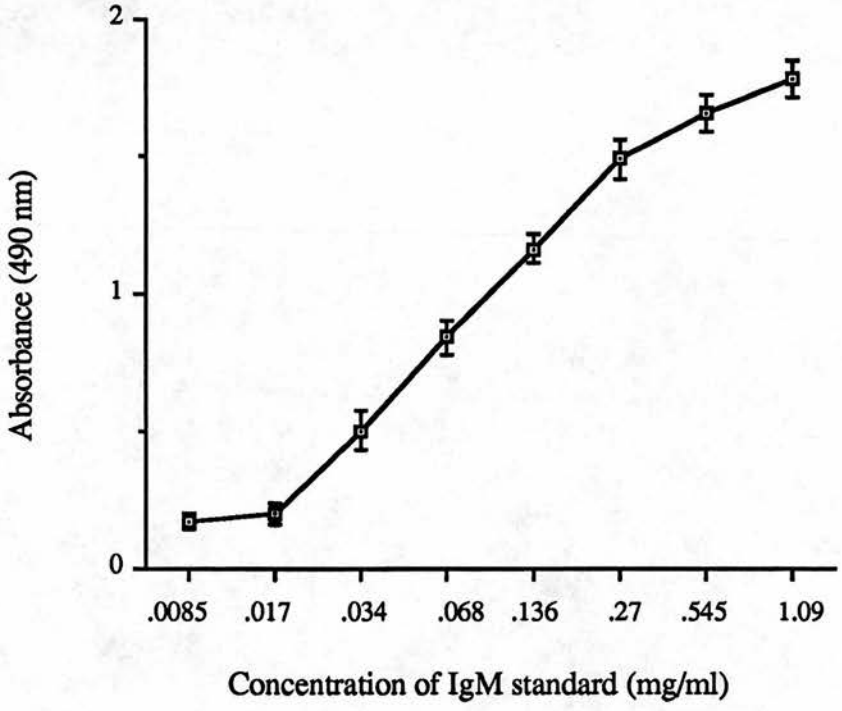

Figure 3.22: Optical density of ELISA for detection of IgM in the serum standard. Each point represents a mean ± SEM of 5 replicates.

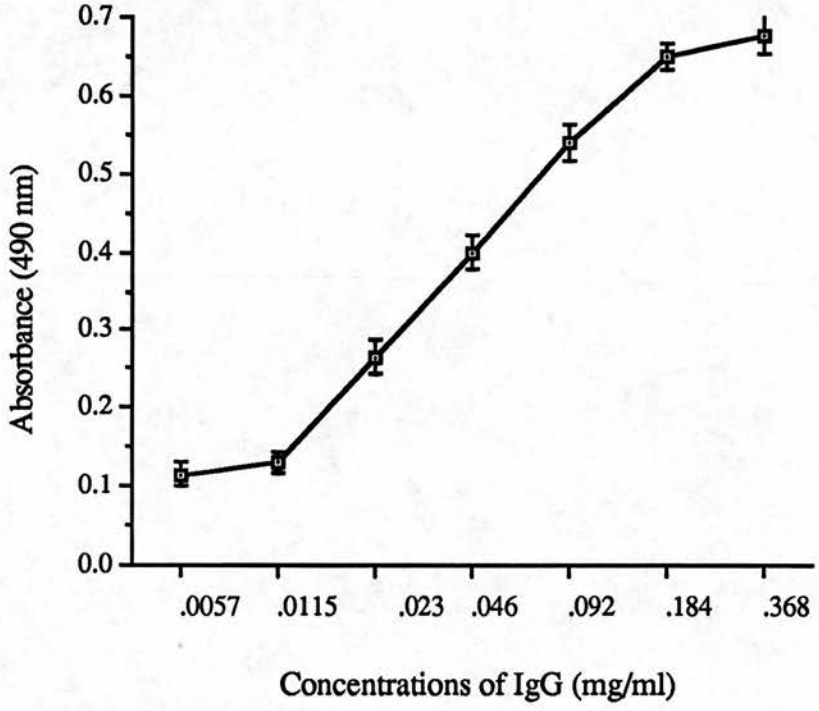

Figure 3.23: Optical density of ELISA for detection of IgG in the serum standard. Each point represents a mean ± SEM for 5 replicates.

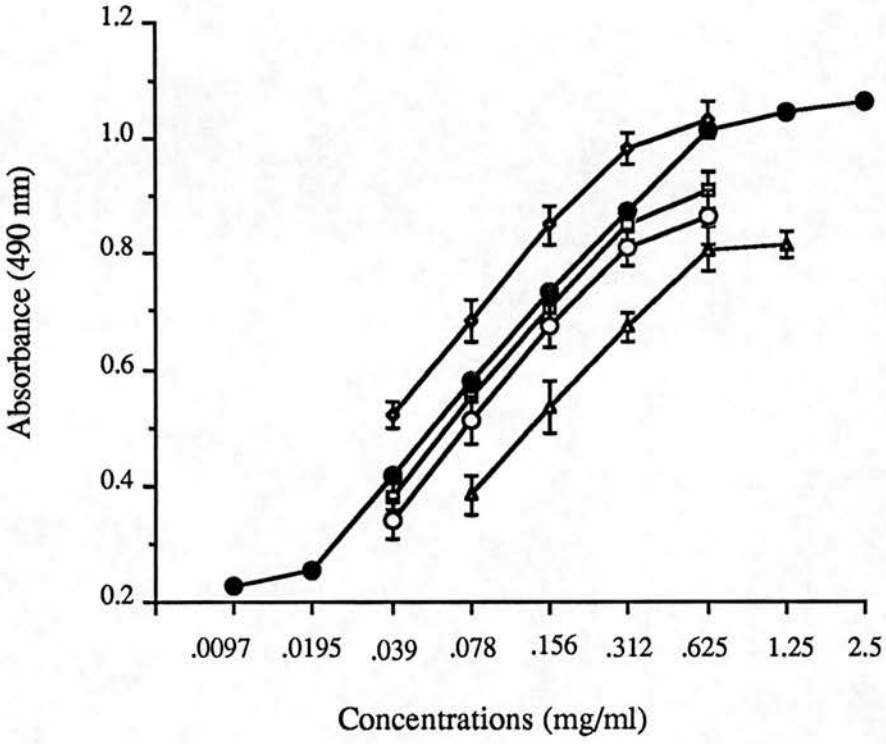

Figure 3.24: The linearity of total serum IgA antibodies of four samples compared to the standard (●). Each point represents the mean ± SEM of 4 experiments.

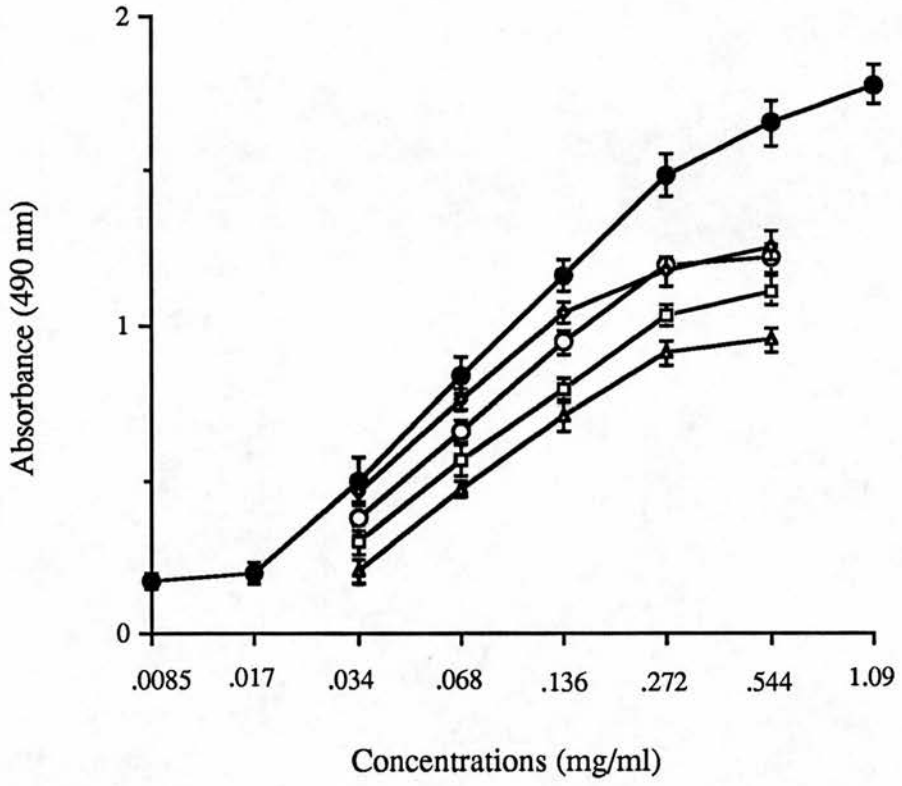

Figure 3.25: The linearity of total serum IgM antibodies of four samples compared to the standard (●). Each point represents the mean ± SEM of 4 experiments.

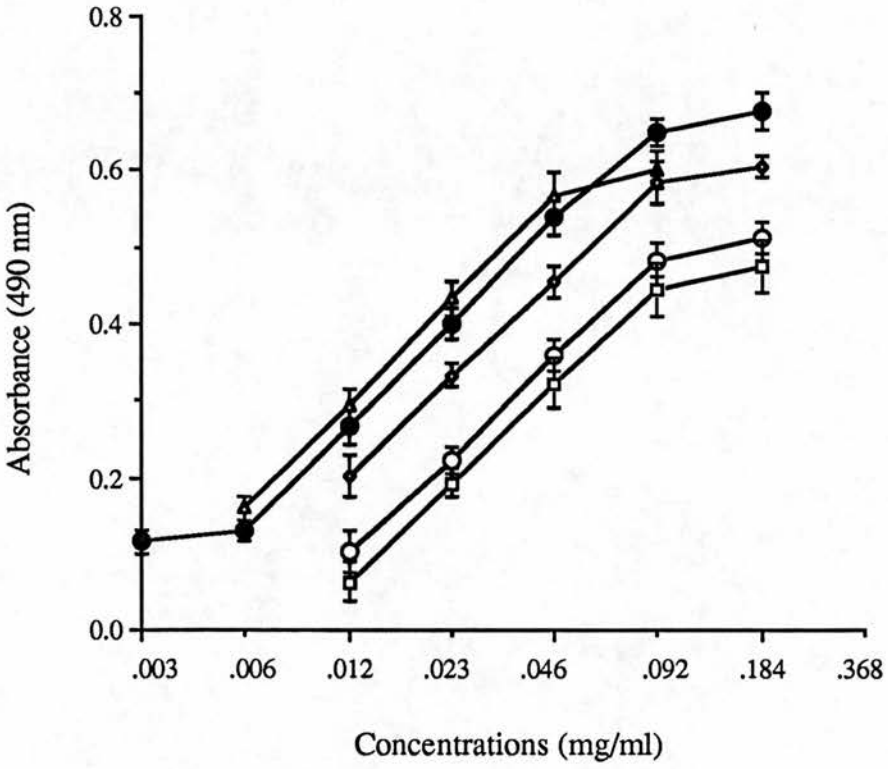

Figure 3.26: The linearity of total serum IgG antibodies of four samples compared to the standard (●). Each point represents the mean ± SEM of 4 experiments.

Table 3.1: Coefficient of variance (CV) values for four readings at different dilutions for the measurement of the three isotypes. The 4 randomly chosen samples were compared with the standard curve.

Samples	Concentration in mg/ml *	C.V. (%)
IgM		
pool	15.1 ± 0.311	2.0
1	20.8 ± 0.327	1.6
2	8.80 ± 0.326	3.7
3	4.75 ± 0.180	3.8
IgA		
pool	9.50 ± 0.212	2.2
1	7.75 ± 0.296	3.8
2	17.5 ± 0.319	1.8
3	12.2 ± 0.432	3.5
IgG		
pool	7.40 ± 0.212	2.9
1	10.3 ± 0.303	2.9
2	4.90 ± 0.204	4.2
3	5.10 ± 0.259	5.1

* Mean of five replicate ± 1S.D.

3.3.8 Reproducibility

Intra-assay variation was measured by testing eight samples including the pool run in six replicates on the same occasion at a dilution of 1/2000 on the same plate with a dilution curve for the standard serum. The mean (range) CV for intra-assay was 2.216% (1.496 - 3.01%), 1.797% (0.863 - 2.644%) and 3.475% (2.255 - 5.844%) for IgA, IgM and IgG respectively.

Interassay variation of the same eight samples was examined on six occasions and titrated against the standard serum. The mean (range) CV for interassay was 2.987% (1.558 - 3.866%), 3.039% (1.04 - 6.135%) and 5.029% (3.062 - 7.324%) for IgA, IgM and IgG respectively.

3.3.9 Specificity of the assays

The specificity of the assays for total and specific antibodies were determined as outlined in section 3.2.1.5.

3.3.9.1 Binding of HRP conjugates antibodies

The specificity of the capture assay was tested by adding the three conjugates at the appropriate step to different wells in the IgA or IgM capture antibody assay. Table 3.2 demonstrates that high OD were obtained only with the corresponding HRP labelled antibody.

3.3.9.2 Specificity of anti-meningococcal antibodies

The specificity of the assay for detection of antibodies to meningococci has been determined for the outbreak strain B:4:P1.15 in three different ways.

Binding of antibody to B:4:P1.15 after absorbing the serum with the outbreak strain greatly reduced the binding for IgA and IgG but less so IgM. Absorption with NG:4:-

reduced the binding by over 50% for IgA and IgG whereas binding was reduced by 36.3% for IgM antibodies. Absorbtion with *N. lactamica* reduced the binding by over 50% for IgA and about 30% for IgG and IgM (Figure 3.27). Absorption of human sera with a mixture of bacteria used in the study virtually abolished the binding of IgG (Figure 3.28).

3.3.9.3. OMP profile obtained on SDS-PAGE

SDS-PAGE analysis of the crude "Sarkosyl" extract produced a profile typical of Gram-negative OMPs; there were two major OMPs and a further minor proteins (Figure 3.29). The two major outer membrane proteins identified in this preparation are identical to those shown by (Tsai, *et al.*, 1981; Frasch *et al.*, 1985; 1986).

Adsorption of serum with OMPs reduced the binding of antibodies to bacteria by approximately 55% for IgG and IgM, definite reduction was found with IgA of about 77% (Figure 3.30).

3.3.10 Comparison between ELISA and SRID

The comparison between ELISA and SRID methods for 16 samples of widely differing immunoglobulin concentration is shown in Figures 3.31 - 3.33. Total IgA, IgG and IgM were measured in μg/l by ELISA and SRID assays. There was significant correlation between both assays: IgA r= 0.97; IgG r=0.94; IgM r=0.98. The ELISA value for serum was significantly greater than the SRID values. Significance of differences between two detection systems were calculated using Student's t-test (two tailed): IgA $p < .0001$; IgM; $p < .0008$; IgG < 0.0449.

3.4 Discussion

The aim of this study was to develop an ELISA system using whole bacteria suitable for the routine detection of an antibody response against *N. meningitidis*. In this study

Table 3.2: The specificity of the capture antibody assay determined by using the three HRP conjugated antibodies.

Plate coated with	Blank	Labelled antibody		
		HRP anti-human IgA	HRP anti-human IG	Anti-human IgM followed by HRP anti-sheep/ goat IgG
anti-IgA	0.058	0.634	0.062	0.066
anti-IgM	0.056	0.059	0.065	0.763

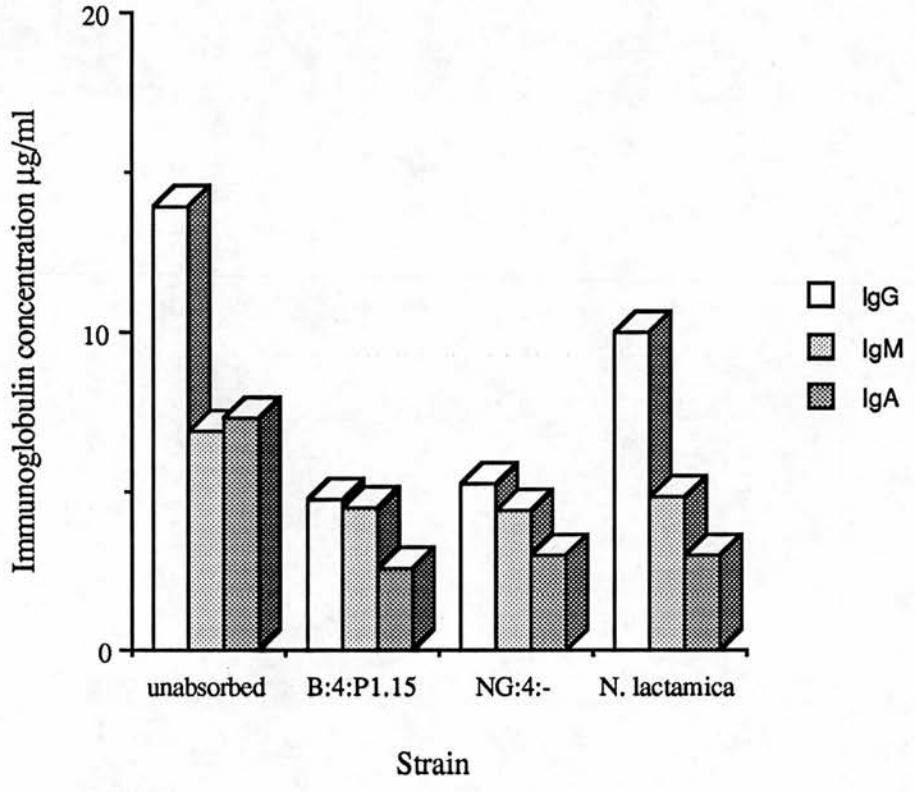

Figure 3.27: The antibody bound to B:4:P1.15 before absorption (unabsorbed) and after absorption of a serum sample with two strains of *N. meningitidis* and *N. lactamica*.

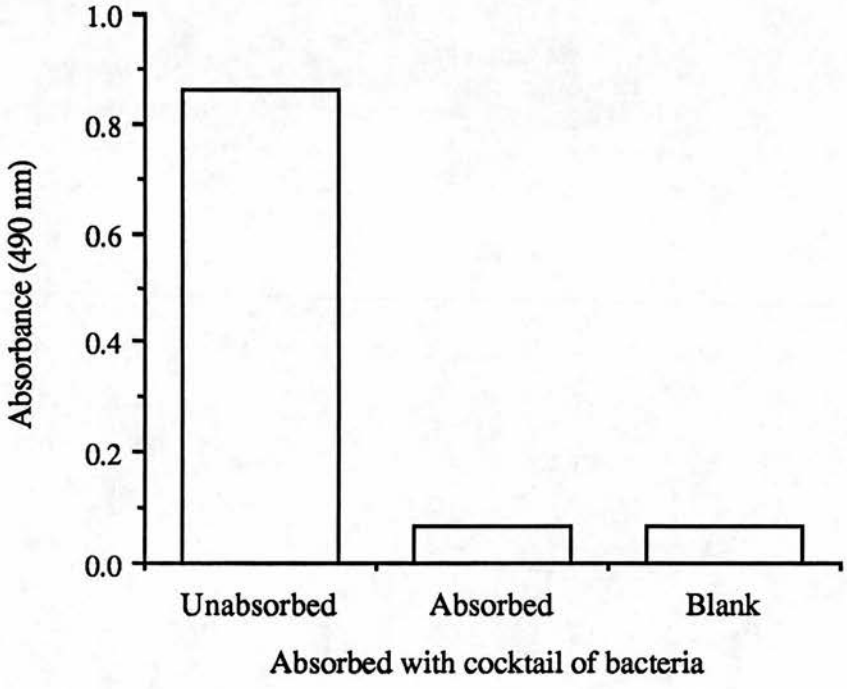

Figure 3.28: Binding of IgG to B:4P1.15 before absorption and after absorption with a mixture of different *Neisseria* isolates. (The blank value = O.D 0.056)

Figure 3.29: Separation of outer membrane preparation of *N. meningitidis* (B:4:P1.15) by SDS-PAGE in a 10% gel stained with Coomassie brilliant blue. The two major outer membrane proteins identified in this preparation are identical to those shown by Tsai *et al.*, 1981.

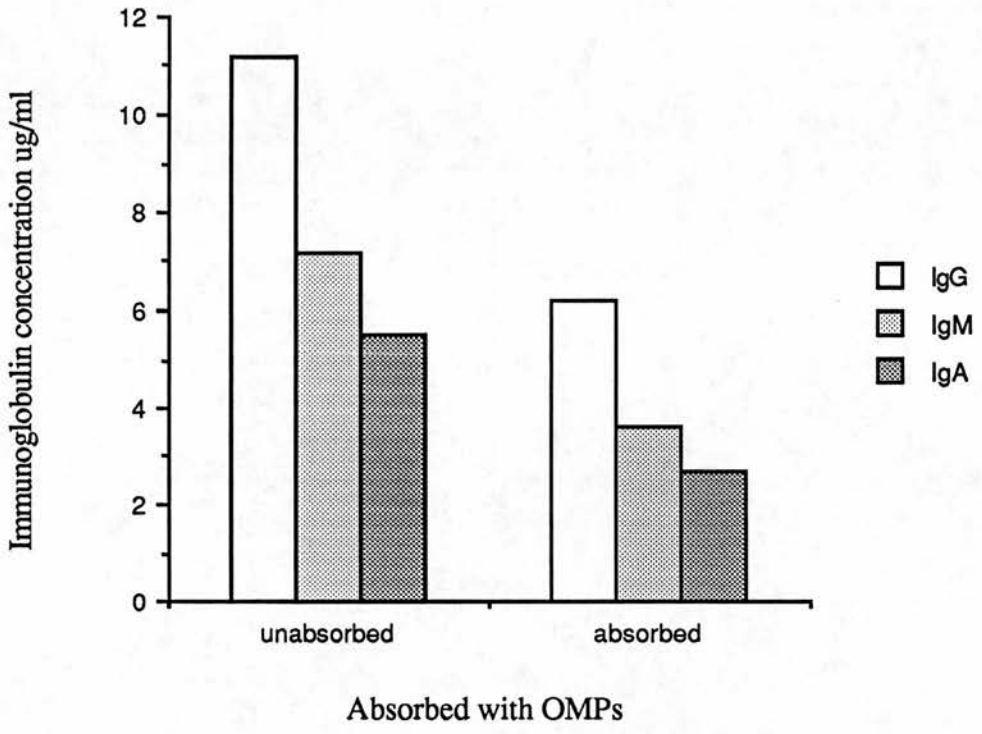

Figure 3.30: Binding of antibody to B:4:P1.15 from an unabsorbed sample and after absorption with OMPs of *N. meningitidis* (B:4:P1.15).

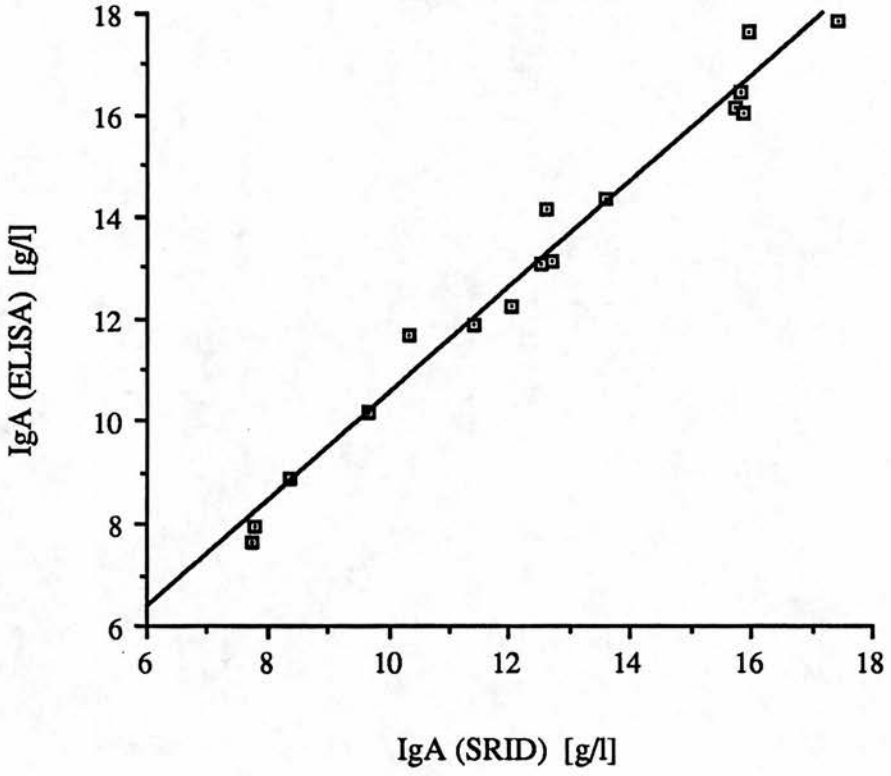

Figure 3.31: Correlation between ELISA and SRID readings for detection of total IgA antibody

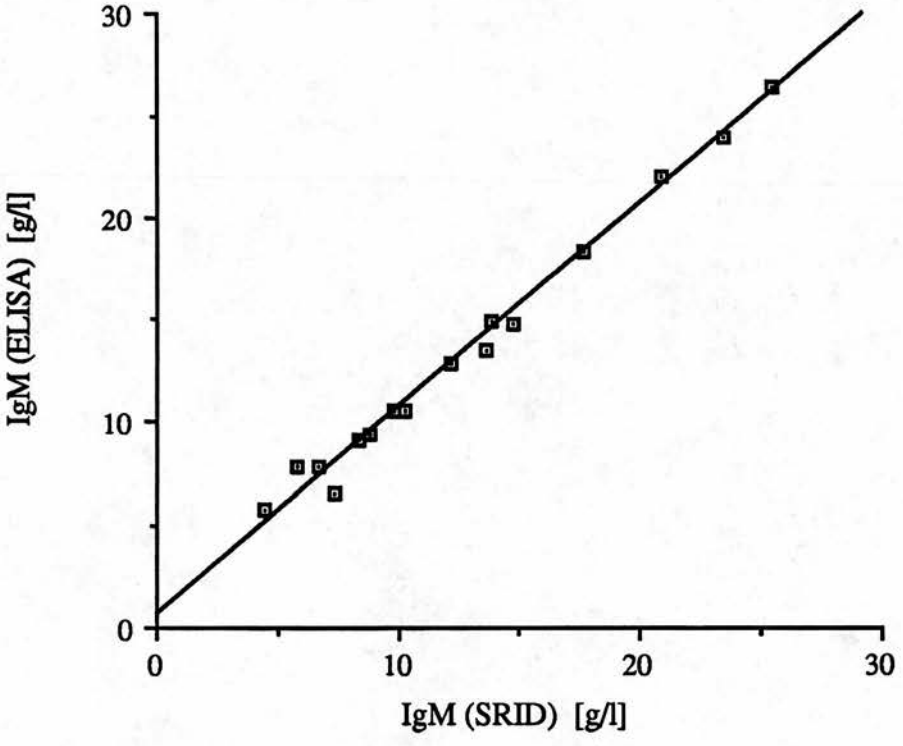

Figure 3.32: Correlation between ELISA and SRID readings for detection of total IgM antibody

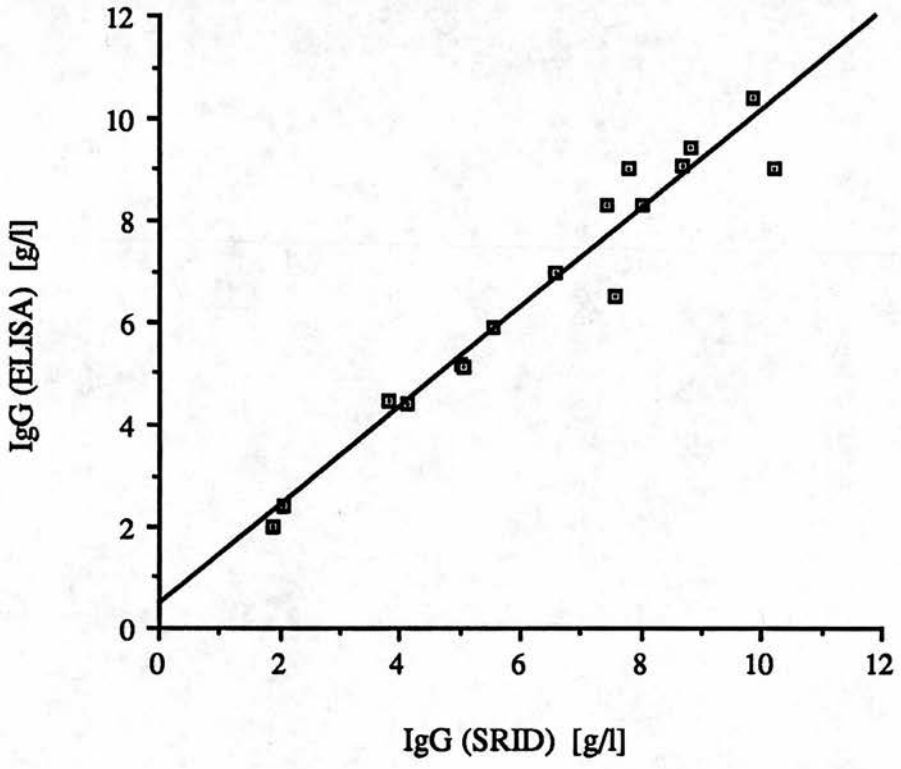

Figure 3.33: Correlation between ELISA and SRID readings for detection of total IgG antibody

an attempt to standardise ELISAs for the quantitation of human IgA, IgG and IgM anti-meningococcal antibodies without the use of a specific standard serum was developed based on the principle of Zollinger and Boslego (1981). The principle of standard-free ELISA is a combination of two simultaneously performed assays; an indirect ELISA for specific (anti-meningococcal) antibodies; and a sandwich ELISA for total immunoglobulin of the antibody class. For this purpose, sandwich ELISAs for quantitation of total IgA and IgM were developed. Optimal concentrations of capture antibody and peroxidase-labelled antibody were determined and the assay modified so that it could be performed simultaneously with the assays for anti-meningococcal antibodies on one micro-test plate.

The sandwich ELISAs for total IgA or IgM immunoglobulins were used to produce reference curves from serial dilutions of a standard serum with known immunoglobulin concentrations. For detection of IgG, the plates were coated directly. The same conjugate was used simultaneously to detect bound immunoglobulin molecules for both total and specific antibodies. Equal enzymatic activity in both assays is assumed to represent identical amounts of the assayed immunoglobulin type. Specific antibody concentrations for different isolates of *Neisseria* were determined from the standard curves for total IgA, IgG or IgM. The standardisation principle can be used to quantitate specific antibodies in individual serum and saliva samples. The standardisation conditions were achieved by the use of identical conjugate, assay conditions and parallel dose-response curves. The advantage of this method is its independence of any specific standard material. Whenever a direct standard cannot be obtained, this method is recommended for the quantitation of specific antibodies from different classes in individual sera and for establishing a standard serum. Zollinger and Boslego (1981) have proposed a procedure for the quantification of specific antibodies by a combination of two radioimmunoassays (RIA).

The sandwich method with a standard containing a known concentration of immunoglobulin of a given class to quantitate the assay was attractive for three reasons. Firstly, since the method essentially standardizes the conjugated-antibody preparation, it is independent of the particular antigen used; therefore it can be applied to a wide range of antigen-antibody systems and to a variety of a solid-phase assays. Secondly, in studies involving the measurement of antibodies in secretions, both total and antigen-specific antibody can be measured in the same assay with the same standard. Finally, since standards for human IgA, IgM and IgG are commercially available, the verification and comparison of data by different laboratories can easily be accomplished.

The use of ELISA for quantitation of total and specific antibodies of a particular immunoglobulin class requires a suitable procedure for optimization of the assay for each antibody class of interest. The ELISA was composed of a blocking step and four incubation steps. All steps and working concentrations were adjusted to obtain the maximum reading while keeping the negative control low. Each interaction step was followed by washing to remove excess reagents and sample constituents. Two reagents are especially important: the solid-phase antibody and the peroxidase-labelled antibody. In the first step, binding of monoclonal antibody to microtitre plate, there is an optimal protein concentration in the coating solution which leads to the formation of a monolayer of antibody molecules on the surface of the wells (Cantarero *et al.*, 1980). The optimum concentration of monoclonal antibodies and whole bacteria for coating the plates were determined by testing different dilutions of these preparations. In this type of ELISA, the concentration of immunoglobulin class in the sample is directly related to the peroxidase-labelled antibody bound to the solid phase after the completion of the ELISA.

There are several commercially available assays for quantitation of normal and low levels of antibodies in human serum. Quantitation of human serum and salivary

immunoglobulins has been performed for many years using radial immunodiffusion techniques (Mancini *et al.*, 1965). This method is based upon the formation of precipitates as serum components are allowed to diffuse in an agar matrix containing monospecific antiserum to human IgA, IgG or IgM. The concentration of immunoglobulin is proportional to the diameter of the ring of precipitation. A small error in such a measurement can result in a large error in the determination of the immunoglobulin concentration. A more sensitive technique would be particularly useful for the measurement of immunoglobulin isotypes which are present in much lower concentration. In addition, radial immunodiffusion analysis requires incubation ranging from 18 h to 3 days.

If the concentration of IgM is high, radial immunodiffusion is the method of choice; however, this method is not of sufficient sensitivity to assay IgM samples in which the IgM concentration is low and a more sensitive method is needed. Among the most sensitive methods, RIA (Jensenius *et al.*, 1983) and ELISA (Dinesen and Saxtrup, 1979; Ishiguro *et al.*, 1982; Smart and Koh, 1983) have been employed for IgM quantitation. Dinesen and Saxtrup, 1979 used two-site solid phase (coated cuvettes) enzyme immunoassay. Ishiguro and colleagues (1982) also used two-site solid phase in which the antiserum was immobilized on silicone rubber pieces. Smart and Koh (1983) used a competitive inhibition enzyme immunoassay. A sandwich design (two-site solid phase) has been used in the present method.

Compared with ELISAs, RIAs, while quite sensitive, have several disadvantages for routine serology. The first disadvantage is the need for relatively expensive counting and monitoring devices as well as adequately trained personnel. Secondly, reagents are expensive and have a short life span.

The ELISA is of comparable sensitivity and specificity compared with counterimmunoelectrophoresis (CIE) precipitin methods. Compared with the CIE method an

ELISA with whole bacteria as antigen, is easier when handling a large number of samples and quantitative results can be obtained.

The aim of this part of the study was to develop a sensitive, precise and accurate method for assessing total immunoglobulin levels and those specific for meningococci in serum and secretions. The data generated and the slopes of the dose response curves were linear for a wide range of immunoglobulin concentrations or serum dilutions. This eliminated a major criticism of using only a single dilution of unknown samples (Challacombe, personal communication); because the dilution slope of the sample is the same as that of the control, the affinity of the sample and control are similar. The CV of linearity was between 1.5 - 5.1% and did not vary between the test sera and the standard.

A number of ELISA tests using whole bacteria as antigen have been developed for epidemiological and diagnostic purposes (Flœgtad *et al.*, 1990). By using whole organisms, one uses a preparation similar to that to which the immune system of the host is exposed. Antibodies to a variety of cell surface antigens might, therefore, be detected. The results of the present study show that adhesion, and more importantly, retention of bacteria to a polystyrene surface can be achieved directly with coating buffer at pH 9.6. This eliminates the need to use either IgG antibodies coated to the plastic surface followed by the immunoadsorption of the corresponding micro-organism or a polyaldehyde derivative (methyl glyoxal) to link the bacteria directly to the solid phase. PBS was ineffective as a coupling agent.

In a parallel trial, antibody levels obtained with the ELISA and a SRID assays of the same aliquot of serum revealed that the ELISA provided significantly higher values. The superiority of the ELISA over the SRID technique lies in its sensitivity.

Evidence of specificity of the ELISA for each isotype was obtained with experiments involving the addition of the three HRP labelled antibodies to separate wells of the same

plate. Those conjugates mismatched with the capture antibodies in the system gave low readings. For instance, on the IgA capture plate, high reading were obtained with corresponding conjugated antibodies (HRP anti-human IgA); however, with other conjugates, the results were the similar to the background reading. This excluded the possibility that other components in serum or saliva could interact with the antigen-binding site of the molecules, either in a cross reactive manner or by non-specific binding. The specificity of the assay was also determined by absorption of the serum with different isolates of *Neisseria* and a mixture of all the strains used in this study. In addition, the specificity was determined by absorption of the serum with OMPs.

The assay has proved sensitive and reproducible and has been satisfactory for all isolates so far examined. The technique for coating the bacteria is easy to perform and to standardize. The method can be applied to a wide range of *Neisseria* isolates and should allow antibodies to particulate antigens to be assayed by solid phase methods.

The ELISA systems described above were used in subsequent studies to investigate the differences in the total and specific immunoglobulin levels, in serum and saliva, between secretors and non-secretors.

Secretor status and humoral immune responses to *N. lactamica* and *N.meningitidis*

4.1 Introduction

Protective immunity to disease due to *N. meningitidis* is associated with the presence of an intact complement system and opsonization or bactericidal antibodies specific for the invading strain (Goldschneider *et al.*, 1969a; b; Lee *et al.*, 1978). These antibodies can be formed through nasopharyngeal carriage of meningococci (Reller *et al.*, 1973). The majority of individuals who develop invasive disease lack protective antibody to the pathogen (Goldschneider *et al.*, 1969a; b). The age range in which meningococcal infection is most prevalent (6 months to 4.5 years) reflects the natural development of antibodies to these pathogens.

The ability of the host to resist infection or colonization by microorganisms is partly dependent on the presence of a fully functional mucosal immune system. Although IgA is the most abundant immunoglobulin in exocrine secretions such as tears, saliva and milk, IgG and IgM are also found in these fluids (Brandtzaeg *et al.*, 1970; Smith *et al.*, 1989). IgM can also function as a true secretory immunoglobulin (Brandtzaeg, 1981), reaching the mucosal surface by the identical secretory-component mediated transcellular pathway that transports IgA. The full protective potential of sIgA is not present in the infant at birth (Berg, 1968). Adult levels of sIgA are not reached until after one year of age (Burgio *et al.*, 1980; Mellander *et al.*, 1984). It has been suggested that in infants the presence of IgM compensates partially for the low levels of IgA (Mellander *et al.*, 1984). IgM to poliovirus and *E. coli* has been found in infants (Mellander *et al.*, 1984; 1986a).

Non-secretors of ABO blood group antigens are over-represented among patients with disease due to *N. meningitidis* (Blackwell *et al.*, 1986a) and in one study among carriers of this bacterium (Blackwell *et al.*, 1990). The lower immunoglobulin levels found in non-secretors compared with secretors have been used to explain the increased susceptibility of non-secretors to rheumatic fever and rheumatic heart disease (Glynn *et al.*, 1956; Clarke *et al.*, 1960). In later studies, non-secretors were reported to have lower levels of both serum (Grundbacher, 1972) and salivary (Waissbluth and Langman, 1971) IgA. It was suggested that specific immune responses at mucosal surfaces of non-secretors might be compromised compared with that of secretors.

The objectives of this part of the study were to use an ELISA system to examine the following questions:

1) Is there a differences in the amount of total serum and salivary IgA, IgG and IgM of secretors compared with non-secretors?
2) Is there a difference in the levels of isotypes specific for *N. lactamica* and *N. meningitidis* in serum or saliva of secretors and non-secretors?
3) Is there a correlation between levels of specific anti-meningococcal antibodies in secretions and in serum?
4) Does recent carriage of meningococci affect the levels of antibody to these bacteria found in secretors and non-secretors?

4.2 Materials and Methods

4.2.1 Determination of immunoglobulin antibodies in serum and saliva by an ELISA technique

Sera and saliva specimens (357) were obtained from staff and pupils (most within the 12-18 years age group) of a school in which there was an outbreak of meningococcal disease.

4.2.1.1 Determination of total immunoglobulin isotype levels.

The total amounts of IgM and IgA were assayed by a capture ELISA method. IgG from serum and saliva was coated directly onto ELISA plates. For the measurement of total IgM and IgA, the wells of polystyrene microtitre plates were coated overnight at 4 °C with either 100 µl of mouse monoclonal anti-human IgM (1/500) or mouse monoclonal anti-serum IgA (1/500) diluted in coating buffer. The coated plates were washed three times with washing buffer and blocked with blocking buffer for 30 min. After washing, 50 µl of serum (1/2000) or saliva (1/10) diluted in blocking buffer were added and incubated for 2 h at room temperature. Plates for detecting IgM were washed and 50 µl of sheep µ-chain specific anti-human IgM (1/80) were added. After washing, 50 µl of HRP donkey anti-sheep/goat IgG (1/20) were added to the plates for 1 h. For IgA plates, 50 µl of HRP rabbit α-chain specific anti-human IgA (1/500) were added to the plates and incubated for 2 h.

Finally, the plates were washed and 50 µl phosphate citrate buffer containing the substrate O-phenylenediamine (0.4 mg/ml, pH 5.0) activated by 40 µl H_2O_2 (30% v/v) were added. The colour change was stopped after 20-30 min by adding 50 µl of H_2SO_2 (12.5%).

To determine total IgG, 100 µl of serum (1/30000-1/50000) or saliva (1/2) diluted in coating buffer were added to the microtitre plates and the plates incubated overnight. After washing, 50 µl of HRP sheep anti-human IgG (1/20) diluted in blocking buffer were added for 2 h. The plates were then treated as for IgA and IgM.

Optical density at 490 nm was determined by an ELISA plate reader (Dynatech) and corrected by subtracting the O.D. of the corresponding blank. A series of twofold dilutions of standard human serum containing known concentrations of immunoglobulins (mg/ml) were tested with the specimens from the study. Samples were tested in duplicate and the readings averaged. ELISA readings were converted to

mg/ml by extrapolating from the curve that was constructed from the readings obtained with standard human serum: IgG = 1140 mg/dl; IgA = 250 mg/dl; IgM = 114 mg/dl.

4.2.1.2 Whole cell ELISA for detection of antibodies to *Neisseria*

Sera and saliva were examined for anti-meningococcal antibodies by whole cell ELISA (section 3.3.2). *N. meningitidis* and *N. lactamica* were cultured overnight on MNYC agar at 37 °C. Large batches of microtitre plates were coated with bacteria to minimise variations due to different antigen preparations and coating procedures. Plates were coated overnight at 4 °C with 100 µl of one of the six bacterial isolates (4.8×10^8 bacteria /ml). The plates were washed and blocked with blocking buffer. The buffer was removed and the plates washed with washing buffer. Undiluted serum or saliva (50 µl) was added to the wells and incubated at room temperature for 2 h. The assay was continued in the same way as the ELISA for total antibodies. The assays for total and specific antibodies were determined at the same time under the same conditions.

4.2.2 Statistical Methods

The statistical analysis of the data was performed with the package SPSS/PC+. The results were summarised by geometric means since the logarithmic values were more normally distributed than the raw data. The significance levels for differences between groups were examined with the Mann-Whitney U test and a P value of 0.05 was regarded as significant. The association between levels of antibodies in serum and in saliva was assessed by Spearman rank correlation.

4.3 Results

A total of 39 variables was recorded for each subject, including: secretor status, carriage of meningococci, smoking habit, total serum and salivary IgA, IgM and IgG and specific IgA and IgM to six different isolates of *Neisseria*.

4.3.1 Total IgA, IgG and IgM antibodies of secretors and non-secretors

The results of the serum and salivary immunoglobulin determinations are shown in Tables 4.1 - 4.5. There was no marked difference in total immunoglobulin levels in sera from secretors compared with non-secretors. Non-secretors had higher levels of the serum IgM antibody compared with secretors, but the difference was not statistically significant. There were differences in the levels of IgA or IgG in the saliva from secretors compared with non-secretors; but, there was significantly more total IgM in the saliva of secretors ($p = 0.0274$) (Table 4.4).

There was no difference in serum or salivary immunoglobulin levels of smokers (37) compared with non-smokers (320) ($p > 0.05$).

4.3.2 Specific immune responses to *Neisseria* species

The results of the specific immune responses to the *Neisseria* species tested are shown in Tables 4.1 - 4.5. There was no marked difference in antibody levels to the isolates in sera of secretors compared with non-secretors, except for IgM to the non-groupable serotype 4 isolate (Table 4.3).

There were significant differences in the mean specific salivary IgM immunoglobulin levels of secretors and non-secretors. Non-secretors had significantly lower levels of IgM for *N. lactamica* and each of the meningococcal isolates tested (Table 4.4), the highest levels of IgM were observed for the NG:4 isolate. Statistical comparison of IgA and IgG antibody levels was also performed, but there was no difference between secretors and non-secretors (Table 4.2 and 4.5).

4.3.3 The correlation between serum and salivary antibodies

Analysis by the Spearman correlation test found no correlation between the levels of IgG, IgA and IgM antibodies in serum and saliva ($p > 0.05$). This suggests that the

Table 4.1: Geometric mean levels of total and specific serum IgA of secretors and non-secretors.

IgA	non-secretors	secretors	p
serum	**n = 107**	**n = 169**	
total IgA	1.34	1.29	0.30
specific			
N. lactamica	14.0	13.0	0.72
B:15:P1.16	12.0	10.0	0.38
C:4	13.0	10.0	0.35
B:4	12.0	11.0	0.76
NG:4	17.0	16.0	0.98
B:4:P1.15	7.1	7.5	0.75

units are for mean total serum IgA is expressed in mg/ml and specific IgA in μg/ml.

Table 4.2: Geometric mean levels of total and specific salivary IgA of secretors and non-secretors.

IgA	non-secretors	secretors	p
saliva	**n = 128**	**n = 205**	
total IgA	0.37	0.42	0.13
specific			
N. lactamica	3.4	3.6	0.56
B:15:P1.16	4.0	3.8	0.85
C:4	3.7	3.4	0.63
B:4	3.4	3.5	0.67
NG:4	7.2	7.3	0.98
B:4:P1.15	9.5	7.1	0.12

units are for mean total salivary IgA is expressed in mg/ml and specific IgA in μg/ml.

Table 4.3: Geometric mean levels of total and specific serum IgM of secretors and non-secretors.

IgM	non-secretors	secretors	p
serum	**n = 107**	**n = 171**	
total IgM	1.27	1.08	0.05
specific			
N. lactamica	9.7	7.7	0.12
B:15:P1.16	10.5	8.5	0.15
C:4:-	6.0	5.3	0.25
B:4:-	22.4	14.7	0.11
NG:4:-	26.4	22.2	0.03
B:4:P1.15	5.2	4.8	0.48

units are for mean total serum IgM are expressed in mg/ml and mean specific IgM in μg/ml.

Table 4.4: Geometric mean levels of total and specific salivary IgM of secretors and non-secretors.

IgM	non-secretors	secretors	p
saliva	**n = 129**	**n = 205**	
total IgM	63.0	87.0	0.027
specific			
N. lactamica	1.8	2.5	0.0000
B:15:P1.16	2.1	3.1	0.0000
C:4	2.9	3.6	0.040
B:4	2.2	3.5	0.0000
NG:4	4.7	5.9	0.017
B:4:P1.15	1.9	2.9	0.0008

units are for mean total and specific salivary IgM levels are expressed in μg/ml.

Table 4.5: Geometric mean levels of total and specific IgG of secretors and non-secretors.

IgG	non-secretors	secretors	p
serum	**n = 103**	**n = 170**	
total IgG	8.22	7.21	0.29
specific			
N. lactamica	5.4	5.3	0.55
B:15:P1.16	5.4	4.2	0.64
C:4	4.5	4.5	0.90
B:4	4.1	3.4	0.61
NG:4	4.6	4.6	0.44
B:4:P1.15	4.4	4.3	0.51
saliva	**n = 36**	**n = 48**	
total IgG	3.9	4.2	0.46

units are for mean total serum IgG levels are expressed in mg/ml and total salivary and specific serum IgG levels in μg/ml.

Table 4.6: Geometric mean of immunoglobulin levels of carriers and non-carriers

isotype	strain	Mean immunoglobulin levels (μg/ml)				
		carriers	(n)	non-carriers	(n)	p
serum						
IgA	C:4	18.0	(67)	13.0	(209)	0.03
IgA	B:4:P1.15	9.5	(66)	8.4	(207)	0.02
IgM	*N. lactamica*	14.4	(68)	7.1	(210)	0.0000
IgM	B:15:P1.16	13.8	(68)	8.2	(210)	0.0002
IgM	C:4	12.4	(68)	4.1	(210)	0.0000
IgM	B:4:P1.15	8.3	(66)	6.5	(207)	0.0025
IgG	B:4:P1.15	7.2	(64)	6.1	(207)	0.0033
IgG	C:4	5.3	(68)	4.2	(205)	0.01
total IgG*		8.28	(68)	7.36	(205)	0.03
saliva						
IgA	NG:4	9.7	(89)	6.6	(243)	0.01
IgM	NG:4	6.5	(89)	5.1	(243)	0.03

* = unit for mean total serum IgG are expressed in (mg/ml)

Table 4.7: Geometric mean of salivary IgM levels of secretors and non-secretors among non-carriers

	Mean immunoglobulin levels (μg/ml)				
	non-secretors	(n)	secretors	(n)	p
Total **IgM**	43.4	(86)	90.05	(158)	0.0007
N. lactamica	1.83	(86)	2.60	(158)	0.0004
B:15:P1.16	2.18	(86)	3.09	(158)	0.0013
C:4:-	5.01	(86)	5.87	(158)	0.135
NG:4:-	4.38	(66)	5.46	(207)	0.0039
B:4:-	2.26	(86)	3.60	(210)	0.0000
B:4:P1.15	1.99	(73)	2.84	(210)	0.0314

Table 4.8: Geometric mean of salivary IgM levels of secretors and non-secretors among carriers

	Mean immunoglobulin levels (μg/ml)				
	non-secretors	(n)	secretors	(n)	p
Total **IgM**	1.5	(45)	1.9	(52)	0.63
N. lactamica	2.29	(43)	3.61	(49)	0.01
B:15:P1.16	3.07	(43)	4.84	(49)	0.001
C:4:-	3.79	(43)	4.55	(49)	0.22
B:4:-	3.29	(43)	4.94	(49)	0.005
NG:4:-	7.39	(34)	8.80	(32)	0.14
B:4:P1.15	2.83	(43)	4.43	(49)	0.09

increased levels of secretory IgM found among secretors are due to locally produced immunoglobulins.

4.3.4 Carriage of meningococci and immunoglobulin levels

Compared with non-carriers, carriers of meningococci had significantly higher levels of antibodies to some isolates in serum and saliva (Table 4.6); however, analysis with respect to both secretor status and carriage revealed that immunoglobulin levels still differed according to secretor status when carriage was taken into account. Among the non-carriers, IgM in saliva was significantly higher in secretors, both total salivary IgM ($p < 0.001$), and specific salivary IgM: *N. lactamica* ($p < 0.001$); B:4:P1.15 ($p < 0.05$; B:4:- ($p < 0.001$), B:15:P1.16 ($p < 0.001$) and NG:4:- ($p < 0.05$) (Table 4.7). Among the carriers, IgM in saliva was significantly higher in secretors for *N. lactamica* ($p = 0.01$), B:15:P1.16 ($p = 0.001$) and B:4:- ($p = 0.001$) (Table 4.8).

4.4 Discussion

The results provided evidence to answer the questions posed in the introduction: (1) There was no marked difference in total immunoglobulin levels in sera from secretors and non-secretors. (2) There was no difference between secretors and non-secretors in total or specific levels of salivary IgA or IgG; however, non-secretors had statistically significantly less total IgM in their saliva compared with secretors. These differences were also observed for salivary IgM to *N. lactamica* and to five isolates of meningococci expressing different combinations of serogroup, serotype and subtype antigens. (3) There was no correlation between levels of serum IgM and secretory IgM in saliva, suggesting that salivary IgM is locally produced and has not leaked from the serum. (4) Although serum and saliva of carriers had significantly higher levels of antibodies to some of the *Neisseria* isolates, the effect of secretor status on IgM remained after adjustment for the effect of carriage.

The association between secretors status and antibody levels has been investigated by a number of researchers, often with conflicting results. Many of these studies failed to look at the factors that might influence results: serum compared with secretory antibodies; total isotype levels; specific isotypes levels; the effect of carriage; the effect of smoking; type of saliva used whether stimulated or unstimulated. Some of the discrepancies might be attributable to the fact that previous studies did not take into account all the factors outlined above; many studies have used only serum in their study (Grundbacher and Shreffler, 1970; Grundbacher, 1972; Blackwell *et al.*, 1986b). Most of the previous studies measured total amounts of immunoglobulin in serum and saliva (Grundbacher, 1972; Blackwell *et al.*, 1989a). Waissbluth and Langman (1971) measured total IgA, IgG and IgM in serum and IgG and IgA in saliva; but they did not measure IgM in saliva. The measurement of specific immune response and the effect of carriage is important; Grundbacher (1972) measured total antibodies only to explain the association between non-secretion with susceptibility to rheumatic fever and rheumatic heart disease. This present study covered the factors identified so far that might influence antibody levels (smoking and carriage) and measured total and specific levels of the three classes of antibody for strains with different antigenic phenotypes. The ELISA was more sensitive than SRID technique used in other studies (Waissbluth and Langman 1971; Grundbacher 1972; Blackwell *et al.*, 1987).

Lower levels of both serum (Waissbluth and Langman, 1971) and salivary IgA (Grundbacher, 1972) were reported for non-secretors, suggesting that specific immune responses at the mucosal surfaces of non-secretors might be compromised compared with secretors. Blackwell and colleagues (1989a) did not confirm this earlier observation with SRID; higher levels of IgA were associated with the presence of meningococci in the individuals from whom the saliva was obtained.

Two recent studies provided evidence of the effects of smoking on local immunity in a case-controlled study of meningococcal carriage during an outbreak of meningococcal

disease. Smoking significantly increased the carriage rate; however, secretory antibodies to meningococci were not measured (Stuart *et al.*, 1989; Blackwell *et al* 1990; 1992a).

Diminished local antibody production might account for the increased carriage rate. The effects of smoking on the concentrations of salivary immunoglobulin isotypes have been investigated in many studies, with conflicting results. Barton and colleagues (1990) found that smokers have higher levels of IgM and lower IgA in saliva. In contrast Olson and colleagues (1984) who used the SRID technique and this study using ELISA found no difference between smokers and non-smokers. The small number of smokers in the present study are considered to be light smokers because of the age range sampled (most within the 12-18 years age group). In addition, the type of saliva is important; whole saliva was used in this study and in Olson's work (1984), whereas Barton and colleagues (1990) used parotid saliva.

There is a large literature on the influence of ageing on systemic immune parameters but few studies of its effects on secretory immunity. In a small study, using the SRID technique with unstimulated saliva, total salivary IgA and IgG levels were not different between 10 young and 10 old subjects; IgM was not detected (Ganguly, 1987). Others have demonstrated a fall in total IgA concentration in nasal secretion with age (Alford, 1968). IgA subclasses in parotid saliva did not change with aging in a comprehensive study (Smith *et al.*, 1987). Salivary antibodies become detectable in the neonatal period, and slowly mature to adult levels. There is no conclusive evidence of any changes in salivary antibodies in a healthy aged population (Barton, 1992).

A major biological role of high molecular weight, polyvalent IgA and IgM secretory antibodies might be to provide a first line of defence against particulate and polyvalent antigens such as bacteria. Individuals with IgM deficiency appear to be at risk of disseminated meningococcal disease (Hobbs *et al.*, 1967; Jones *et al.*, 1973). It has

been demonstrated that locally produced IgM in IgA-deficient patients had anti-viral activity (Ogra *et al.*, 1974). The lower levels of sIgM in non-secretors might contribute to susceptibility to colonization, particularly among infants under the age of 12 months in whom sIgM is the major class of antibody on mucosal surfaces. The presence of sIgM in early infancy has been suggested to compensate for the absence of sIgA (Mellander *et al.*, 1984). IgM has been detected in saliva of infants who were as young as one month of age (Gleeson *et al.*, 1982). If sIgM provides a crucial host defence during this period of life the lower levels of sIgM found for non-secretors might contribute to their apparent susceptibility to meningococcal disease.

The carrier state and the subsequent natural immunization to meningococci that occurs are logical starting points in investigating the human immune response to this organism. The method developed here and the results will be useful in examination of serum and secretory response to antigens under consideration as candidate vaccines.

Inhibitory effect of saliva on attachment of bacteria to epithelial cells

5.1 Introduction

The ability of a number of bacterial pathogens to cause disease is associated with their ability to attach to mucosal surfaces (Smith, 1977; Silverblatt and Cohen, 1979; McGee *et al.*, 1988). Although non-specific factors such as surface charge, pH, ionic bridging and hydrophobic interactions might be important (Watt and Ward, 1980), specific adherence of microorganism to epithelial cells is an important step in the colonization process.

Early investigators of meningococcal pathogenesis identified the nasopharynx as the natural habitat of these bacteria. This was suggested to be the site from which the organism was transmitted to other individuals (see Stephens and Whitney, 1985). The meningococcal carrier state and, most likely, meningococcal disease result from the initial colonization of nasopharyngeal mucous membranes by *N. meningitidis*. Once colonization is established, internalization of the bacteria by epithelial cells is possible (Stephens, 1989).

Colonization of mucosal surfaces by bacteria is in part determined by the capacity of the host to block attachment to specific receptors on epithelial cells. The first line of defence is the immunoglobulins, primarily secretory IgA, and high molecular weight glycoconjugates present on the mucosal surface that prevent the interaction of bacterial adhesins with complementary host cell receptors (Gibbons and van Houte, 1975; Beachey 1981).

As this present study showed that non-secretors had lower levels of total IgM and IgM specific for *Neisseria*, a flow cytometric assay was developed to assess the effect of

salivary antibodies on the attachment of meningococci to epithelial cells. The following questions were examined:

1) Does saliva contain factors that might inhibit the attachment of meningococci to buccal epithelial cells (BEC)?

2) Is there any difference between saliva specimens from secretors and non-secretors in their ability to inhibit binding of meningococci to epithelial cells?

3) Is saliva from which specific antibodies have been absorbed still capable of inhibiting the attachment of meningococci?

4) Is salivary IgA the prime factor in the inhibition of bacterial binding to epithelial cells?

5.2 Materials and Methods

5.2.1 Antibody source

5.2.1.1 Pool

Two pools of saliva, one from secretors and one from non-secretors, were prepared from material collected in the survey of school children (section 2.8.2). Each pool was divided into four aliquots. The first was not treated. The second was absorbed with a mixture of bacteria (absorbed). The third was absorbed with the bacteria then filtered as outlined below (absorbed/filtered). The fourth was used for the preparation of purified IgA and IgM (section 2.8.4).

5.2.1.2 Absorption of the pool

A 30 ml. aliquot of the pool was absorbed with the washed sediment of bacterial suspension containing > 10^{10} bacteria for 2 h at 37 °C with shaking followed by a further 24 h at 4 °C without shaking. The bacteria were removed from the saliva by

centrifugation at 1500 g for 20 min. This procedure was repeated three times. Each absorption was carried out with a mixture of the *Neisseria* strains used in this study. This was done to remove any reactive antibodies present.

5.2.1.3 Absorption and filtration of the pool

The further aliquot of the pool was absorbed as outlined above then filtered initially with a 5 μm bacterial filter (Nucleopore Corporation, Pleasanton). The saliva was then passed through a filter (0.22 μm) with a sterilising Nuflow cellulose acetate membrane (Oxoid Ltd., England). This procedure was performed because any unlabelled components of bacteria remaining after centrifugation might competitively inhibit the attachment of fluorescein-labelled meningococci to epithelial cells.

5.2.2 Quantitation of antibodies

Specific *N. meningitidis* antibodies were measured with the whole cell ELISA (section 4.2.1.2) in the saliva preparations: unabsorbed pool, absorbed pool, absorbed/filtered pool and fresh saliva collected from laboratory staff. Purified IgA and IgM were also measured by the same technique, the purity of each isotype was checked with the ELISA as described in section 4.2.1.2.

5.2.3 Labelling the bacteria

The method used is a modification of that described by Wright and Jong (1986) for labelling bacteria with fluorescein isothiocyanate (FITC) (Sigma). A heavy suspension of bacteria in PBS was prepared from colonies grown overnight on MNYC plates. Bacteria were washed three times in D.PBS+B by centrifugation at 1000 g for 20 min. FITC (400 μg) was dissolved in 1 ml of buffer (0.05 M sodium carbonate + 0.1 M sodium chloride, pH 9.2) and added to the bacterial pellet. The mixture was incubated at 37 °C for 20 min and washed three times with D.PBS+B by centrifugation at 1000 g for 20 min to remove free FITC.

5.2.4 Adherence to epithelial cells

The procedure for the detection of *Neisseria* adhering to epithelial cells is modified from that described by Rahat (1990) and Saadi and colleagues (1993). Initially, four isolates were used; *N. lactamica* and three isolates of *N. meningitidis,* B:4:P1.15, C:4:-, and NG:4:-. After labelling, the bacterial concentration was adjusted by measuring the optical density (section 2.4.5) to produce the required ratios of bacteria to epithelial cells in the final mixture; 300:1, 100:1 and 30:1. Buccal epithelial cells (2.5×10^5) (200 µl) were mixed with 200 µl of bacteria or buffer in a 5 ml tube and incubated at 37 °C for 30 min with continuous shaking. The cells were washed three times with D. PBS+B by centrifugation at 300 g for 10 min and fixed with 1% (v/v) buffered paraformaldehyde. Binding of the bacteria to epithelial cells was analysed with an EPICS 'C' flow cytometer (section 2.8.3). The binding of different isolates of *Neisseria* were compared.

5.2.5 Inhibition of binding assay

N. meningitidis serogroup B:4:P1.15 directly conjugated with FITC was used in this assay. After labelling, the optical density of the bacterial concentration was adjusted to give a bacteria:BEC ratio of 100:1 in the final mixture (section 5.2.4). Equal volumes (200 µl) of bacteria and saliva were mixed and incubated at 37 °C for 30 min with continuous shaking. The mixture was centrifuged at 300 g for 10 min and the saliva removed. D.BPS+B (200 µl) was added to the tube containing pelleted bacteria followed by 200 µl of BEC and incubated at 37 °C for 20 min. The BEC were washed three times in D.PBS+B at 300 g for 10 min. The cells were fixed with 1% buffered paraformaldehyde and analysed by flow cytometry. A control was included each time containing D.PBS+B instead of saliva.

5.2.6 Determination of the effect of FITC on the bacterial surface

To illustrate that the FITC did not alter binding of antibodies to the bacterial surface a whole cell ELISA technique was used.

Separate wells of a 96-well microtitre plate were coated overnight at 4 °C with 100 µl of FITC labelled or unlabelled bacteria (4.8×10^8 bacteria/ml). The plate was washed and blocked with blocking buffer. After washing, undiluted saliva, absorbed saliva, purified IgA and IgM were added to the two types of bacteria. The assay was continued in the same way as for whole cell ELISA (section 4.2.1.2).

5.2.7 Analysis of results

For each sample analysed by flow cytometry two parameters were obtained: 1) the percentage of cells in the population that showed fluorescence above background; 2) the mean channel number for the population of positive cells, reflecting the mean number of bacteria bound to the cells. In some experiments the binding index (BI) for the preparation of cells was calculated by multiplying the percentage of fluorescent cells by the mean channel number for the population of positive cells.

5.2.8 Statistical analysis

For comparison of inhibition of binding of the bacteria by the different saliva preparations, the mean level of fluorescence in the population of cells in each sample was compared with the control by the following formula: % inhibition = 100 - [(mean channel number of the sample + mean channel number of the control) x 100]. The results were analysed statistically by the Student's t-test to calculate confidence intervals. Results were considered significant if $p < 0.05$.

5.3 Results

5.3.1 Attachment of *Neisseria* isolates to BEC

The attachment of *N. lactamica* and three different isolates of meningococci (B:4:P1.15; C:4:- and NG:4:-) to BEC of two individuals was studied. The binding index (BI) of each isolate was determined, and the results demonstrated that the levels of attachment of *N. lactamica* and NG:4:- were similar and were higher than B:4:P1.15 and C:4:- at the three doses of bacteria used (Figure 5.1). The attachment of meningococci to BEC decreased as the ratio of bacteria:BEC decreased. At a ratio of 30:1 the BI for all four strains was low. In subsequent studies the intermediate bacteria:BEC ratio of 100:1 was used. This ratio was considered to be appropriate because any changes could be clearly illustrated; therefore, it should be possible to detect inhibition of attachment under these conditions. At the ratio of 100:1, almost all the cells showed fluorescence above the background (cells not exposed to bacteria). The level was between 99.3-99.7% for the different bacteria used.

5.3.2 Inhibition of attachment of meningococci by saliva

The ability of saliva to inhibit the attachment of *N. meningitidis* B:4:P1.15 to BEC was studied by the flow cytometry technique adopted for this study. BEC and saliva collected from the laboratory staff (section 2.1.2) were also used in this study. The binding of meningococci to BEC after treatment with saliva was compared with the binding of meningococci to BEC treated with D.PBS+B. The mean levels of fluorescence (as determined by mean channel number) in the population of positive cells both in control and treated samples were used as the measurement of binding. At the ratio of bacteria:BEC used, almost all the cells showed fluoresence above the background (cells not exposed to bacteria). The level was usually 99.9% for the control and between 95 - 99.8% for test sample. Treatment of the bacteria with saliva inhibited the attachment of meningococci to BEC. The inhibition was dose dependent

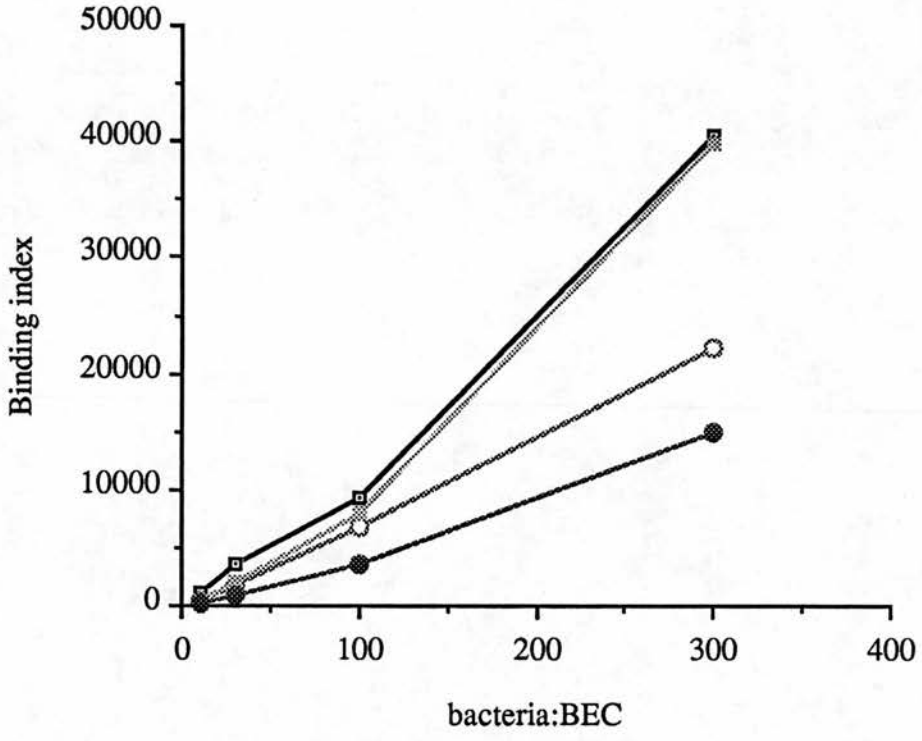

Figure 5.1: Attachment of *N. lactamica* (▫), and three strains of *N. meningitidis*: B:4:P1.15 (●), C:4:- (○) and NG:4:- (▒), to BEC fron one donor. Similar results were obtained with cells from another donor.

(Table 5.1). The highest inhibition was obtained when one volume of bacteria was incubated with two volumes of saliva, the lowest inhibition when 1/2 volume of saliva (diluted with D.PBS+B) was incubated with one volume of bacteria.

5.3.3 Inhibition of attachment of meningococci by saliva from secretors and non-secretors

Tables 5.2 and 5.3 present the assessment of inhibition of bacterial attachment by the pool, fresh saliva and the absorbed/filtered pool preparations from secretors and non-secretors. The BEC and saliva were collected from different subjects (section 2.1.2). Compared with the controls, there was a statistically significant inhibition of binding of strain B:4:P1.15 to BEC by the untreated pool ($p = 0.0002$) and fresh saliva ($p = 0.013$) from secretors and untreated pool ($p = 0.0004$) and fresh saliva ($p = 0.006$) from non-secretors (Tables 5.2 and 5.3). There was no significant difference between the inhibitory activity of the pool compared with fresh saliva for secretors ($p = 0.45$) or non-secretors ($p = 0.24$).

The inhibitory effect was greater for saliva from secretors, in which there were significantly higher levels of inhibition in both the pool ($p = 0.049$) and fresh saliva ($p = 0.0001$) compared with non-secretors. The mean level of fluorescence (determined by mean channel number) of positive cells in control samples varied considerably from day to day.

5.3.4 The effect of absorption of saliva with *Neisseria*

Absorption of antibodies with meningococci was assessed with the whole cell ELISA. The saliva was repeatedly absorbed with bacteria until no antibody in the saliva specific for meningococci was detectable by the ELISA.

The absorbed pool obtained from secretors was tested for its ability to inhibit the attachment of meningococci to epithelial cells. In spite of no antibody being detectable

by ELISA, there was considerable inhibition of attachment of bacteria by the absorbed pools. The percentage of inhibition of binding by this absorbed pool was between 44-60% and the reduction was statistically significant compared with the control containing no saliva ($p = 0.0095$).

The inhibitory activity was further investigated with the absorbed/filtered pool since residual antigen might account for the inhibition; the results were compared with the unabsorbed pool. The percentage of inhibition of bacterial binding was not as marked as that observed with the unabsorbed pool, but it was statistically significant for secretors ($p = 0.018$) and non-secretors ($p = 0.005$). The mean percentage of inhibition of bacteria by the unabsorbed pool and the absorbed/filtered pool was 72% and 18.4% for secretors and 56.5% and 18.1% for non-secretors. There was a significant difference in the inhibition between the pool and the absorbed/filtered pool for secretors ($p =0.0001$) and for non-secretors ($p = 0.018$) (Tables 5.2 and 5.3).

5.3.5 Inhibition of attachment of meningococci by secretory IgA

Antibody-dependent inhibition of binding was demonstrated with purified IgA and IgM obtained from the pool collected from the school population (section 2.8.4). Table 5.4 demonstrates the percentage inhibition of bacterial binding by affinity purified IgA and IgM. The mean percentage inhibition following treatment of bacteria with IgA was 20% and 10% for IgM. Compared with the control, the inhibition of binding was statistically significant for both IgA ($p = 0.02$) and IgM ($p = 0.03$). Inhibition by purified IgA and IgM antibodies was compared to determine which antibody class had more inhibitory activity. There was no statistical difference between IgA and IgM ($p = 0.06$).

The role of antibodies in inhibition of binding was further analysed by testing the inhibitory activity of a panel of saliva preparations from which either IgA, IgM or IgG had been absorbed and comparing results with inhibition observed with whole saliva

Table 5.1: Inhibition of binding of B:4:P1.15 to BEC by different concentrations of saliva.

bacteria: saliva†	mean flourescence of control	percentage inhibition			
		(secretors)±		(non-secretors)±	
		pool	absorbed	pool	absorbed
1:2	74	80	58	75	52
1:1	95	70	46	68	45
1:0.5	95	58	37	53	32

± The percentage of inhibition was calculated from the mean channel number of control and test samples

† The ratio of the volumes of bacteria (concentration of bacteria = 10^7 per ml) to saliva

Table 5.2: Percentage inhibition of adherence of B:4:P1.15 to BEC by different saliva preparations from secretor donors.

Experiments	mean flourescence of control	percentage inhibition ±			
		pool	fresh saliva†	absorbed	absorbed/ filtered
1	80	74	79	55	10
2	273	58	75	60	25
3	254	65	80	ND	28
4	196	92	70	46	28
5	123	92	80	ND	28
6	308	60	62	44	21
7	271	63	61	50	17

± The percentage of inhibition was calculated from the means of the control and test samples.

† Each row represents experiments on different days with BEC and fresh saliva from different individuals

ND Not determined

Table 5.3: Percentage inhibition of adherence of B:4:P1.15 to BEC by different saliva preparations from non-secretor donors

Experiments	mean flourescence of control	percentage inhibition ±		
		pool	fresh saliva†	absorbed/filtered
1	39	60	65	28
2	381	63	54	14
3	138	55	64	24
4	150	45	55	11
5	65	58	51	20
6	406	56	43	12
7	356	59	49	18

± The percentage of inhibition was calculated from the means of the control and test sample.

† Each row represents experiment on different days with BEC and fresh saliva from different individuals

Table 5.4: Percentage inhibition of bacterial adherence of B:4:P1.15 to BEC by purified antibodies and whole saliva

Experiment	mean flourescence of Control	percentage inhibition±						
		untreated pool	fresh saliva	purified IgA	purified IgM	saliva without IgM	saliva without IgG	saliva without IgA
1	107	78	70	19	8	56	49	59
2	132	77	70	24	10	58	53	65
3	116	81	76	ND	ND	55	60	76
4	164	84	72	ND	ND	63	62	57
5	134	80	83	17	12	69	80	77

± The percentage of inhibition was calculated from the means of control and test sample

Each row represents experiment on different days

ND Not determined

(Table 5.4). Compared with the control, each type of saliva demonstrated high levels of inhibition: for saliva without IgA ($p = 0.0007$); for saliva without IgG ($p = 0.0015$); and for saliva without IgM ($p = 0.0002$). There was no statistical difference in inhibitory activity between the three saliva preparations ($p > 0.05$).

5.3.6 The effect of FITC on binding of meningococci

The possibility that the procedure used to label the bacteria with fluorescein altered their surface components was investigated using the ELISA technique. The binding of antibacterial antibodies to FITC labelled and unlabelled bacteria was identical (data not shown).

5.4 Discussion

The results of the experiments provided data to answer the question posed in the introduction. (1) With the flow cytometry technique it could be shown that saliva inhibited the attachment of bacteria to epithelial cells. (2) This inhibition was significantly higher with saliva from secretors than saliva from non-secretors. (3) After absorption of saliva with a mixture of bacteria, the saliva retained high levels of inhibitory activity. Absorbed saliva that had been filtered was still inhibitory, but to a lesser extent compared with the unfiltered absorbed saliva. (4) Significant inhibition of binding was demonstrated with purified IgA and IgM, but there was no statistical differences between IgA and IgM. A panel of saliva preparations from which either IgA, IgM or IgG was absorbed was also examined and each preparation of saliva demonstrated high levels of inhibition.

The majority of studies of inhibition of adhesion of bacteria to epithelial cells *in vitro* have been carried out by light microscopy (Tramont, 1977; McChesney *et al.*, 1982; Taylor *et al.*, 1990). The basic method consists of mixing epithelial cells with bacteria and incubating the mixture for an appropriate period. Excess bacteria are removed by centrifugation, then the cells are resuspended and a drop of this suspension dried on a

glass slide and examined under the light microscope. This assay is severely limited by technical consideration. It is laborious, prone to subjective errors in counting and samples few epithelial cells. These limitations have been overcome in this study by the use of fluoresceinated *N. meningitidis* and analysis of the adherence by flow cytometry. The advantages of flow cytometry are considerable. Over a thousand BEC can be analysed within a few minutes; it is less prone to subjective errors; and, unattached bacteria are not recorded by the instrument.

FITC was used to label bacteria in this study. FITC molecules bind to surface proteins non-specifically. The dye did not interfere with the binding of immunoglobulins since both labelled and unlabelled bacteria bound similar amounts of antibodies when assessed by whole cell ELISA.

This study used saliva obtained from a population in which there was an outbreak due to *N. meningitidis* B:4:P1.15 to demonstrate that secretions contain factors capable of inhibiting the attachment of those bacteria to epithelial cells. A pool of saliva was prepared and divided into four lots: unabsorbed, absorbed, absorbed/filtered and the remainder was used to prepare purified IgA and IgM. Flow cytometry was used for the first time to study the ability of secretions to inhibit the attachment of meningococci to cells.

The initial experiments were simply to determine if the binding assay would provide results similar to those obtained by light microscopy, and to allow determination of appropriate bacteria to cell ratios for subsequent experiments. The results show that increasing the ratio of bacteria to cells increased the level of meningococcal binding. Different patterns of attachment were obtained with different isolates of *Neisseria* ; binding of the non-capsulate *N. lactamica* and non-groupable *N. meningitidis* NG:4:- was greater than that observed for the two capsulate strains of meningococci B:4:P1.15 and C:4:-. Studies by Stephens and McGee (1981) have previously shown that the

non-groupable isolates bound better to epithelial cells than their serogroupable counterparts.

The results demonstrate that treatment with saliva reduced the attachment of *N. meningitidis* to epithelial cells, and this inhibition was dose dependent. Both the pooled saliva from the school children and fresh saliva collected from departmental staff at the time of the experiments showed significant inhibition which suggests that the inhibitory factors did not deteriorate with long term storage at -20 °C. Significantly higher levels of inhibition were observed with saliva from secretors compared with saliva from non-secretors.

The inhibition of binding by the saliva led to an investigation of the effect of absorption of antibodies specific for the bacteria tested. Although after adsorption no antibodies could be detected by whole cell ELISA, the saliva still contained significant inhibitory activity. Absorbed saliva that had been filtered to remove any remaining bacteria or debries was also inhibitory, but it was not as effective as unfiltered absorbed saliva. The possibility that the filtered material might contain some cell envelope fragments can not be eliminated; however, this suggests that the saliva contained inhibitors that are not immunoglobulins and are present in large quantities.

Secretory IgA is the predominant immunoglobulin in the specific immune responses at mucosal surfaces. It has been reported to inhibit bacterial adherence to mucosal surfaces by blocking adherence determinants (Abraham and Beachey, 1985), reduction of negative charge and hydrophobicity of bacteria (Magnusson *et al.*, 1979) and the agglutination of bacteria to which it is specifically directed (Liljemark *et al.*, 1979). Several *in vitro* studies on the protective role of secretory IgA have demonstrated a decreased attachment of *Vibrio cholerae* to intestinal epithelium (Fubara and Freter, 1973), streptococci to oral epithelium (Williams and Gibbons, 1975), *E. coli* to urinary

tract epithelial cells (Svanborg-Eden and Svennerholm, 1978), and *Pseudomonas aeruginosa* to tracheal epithelial cells (Niederman *et al.*, 1986).

Salivary IgA probably contributed to inhibition of binding of meningococci to BEC and was demonstrated by identifying blocking activity in IgA purified from the saliva. The percentage inhibition of bacterial binding by affinity purified salivary IgA was low (mean 20%) compared with the untreated pool (mean 80%); and, the inhibitory effect was also lower compared with saliva containing no antibodies (absorbed) or saliva depleted of IgA, IgG or IgM. The results indicate that salivary IgA plays a minor role in inhibition of binding of meningococci to epithelial cells.

These observations suggest that significant amounts of non-antibody blocking factors are present in the saliva and that these factors can reduce the numbers of bacteria attaching to buccal cells either by binding to bacteria or acting on the epithelial surface in the *in vitro* system examined in these studies. In histological examinations, Stephens and his colleagues (1983a) found that immunoglobulin was rarely associated with meningococci on the mucosal surface. Stephens and Farley (1991) suggested that immunoglobulins normally present in their model might not influence the pathogenic events.

Among the non-immune defences against bacterial adherence in the oral cavity are naturally secreted high molecular weight glycoproteins that might behave as receptor analogues, either aggregating (Williams and Gibbons, 1975; Babu *et al.*, 1986a) or binding to bacteria (Babu *et al.*, 1986b), thereby reducing the number of organisms available to attach to specific receptors. Innate defence factors such as salivary lysozyme have been demonstrated to have aggregating activity (Laible and Germaine, 1982). Blood group antigens can also affect binding to mucosal cells (Blackwell, 1989). Glycoproteins might bind to epithelial cell receptors and block the adherence of organisms (Hasty and Simpson, 1987). An imbalance in any one or combination of

these adherence modulating factors in any subject might be significant in determining adherence patterns of susceptibility to colonization.

The oral cavity poses a complex microenviroment where numerous components in whole saliva make it difficult to assess the significance of any single factor such as the inhibitory effect of salivary IgA on the adherence of *N. meningitidis.*

The variation in the level of bacterial attachment in different control samples and variation from day to day can be explained in a number of ways. Application of shear forces can reduce the number of bacteria attached to cells. Variation in force used to disperse cells at each washing could result in detachment of variable proportions of bacteria (Rasanen, 1981; Christersson *et al.*, 1988). The day to day variation might be partly due to daily calibration of the flow cytometer which requires fine adjustment in the alignment of the laser beam and sample stream with light sensors prior to the experiments. Any changes in the setting can affect the reading. Changes in the cell suspension or cell flow rate in the flow cytometer can also affect the results. In all experiments the cells in suspension were thoroughly dispersed and flow rate was also kept approximately at the same rate. The same conditions were always applied to minimize any changes in the readings. Fluctuations in the binding of bacteria to aliquots from stock samples of buccal epithelial cells have been observed (Tramont, 1977).

The influence of pili on meningococcal adhesion cannot be ignored. Pili have been shown to be important in the binding to a number of epithelial cell types. Their ability to bind to different epithelial surfaces was shown by Stephens and McGee (1981). Olafson and colleagues (1985) indicated that *N. meningitidis* displays considerable intra- and interstrain heterogenicity with respect to both pilus subunit size and antigenicity. This immunologic diversity poses difficulties in assaying these components in attachment and comparing results from different laboratories. The

isolate used in this study was not pilate. The role of pili is discussed in detail in a later section.

The experiments carried out in this chapter used the outbreak strain B:4:P1.15 for the attachment and inhibition by saliva. The results shown in Figure 5.1 indicate that the system is suitable for testing other strains; and the assay might be extended for other strains and species of bacteria.

Further studies of interactions of meningococci with mucosal host defences should provide information about the mechanism by which pathogenic bacteria overcome host barriers to attach to receptors and to colonize mucosal surfaces. Further characterization of processes that block the attachment of *N. meningitidis* is needed to assess this initial step in the pathogenesis of meningitis. The observation that saliva from secretors is more effective in inhibiting attachment to buccal epithelial cells suggests this might contribute to their apparent increased resistance to meningococcal disease compared with non-secretors.

Bactericidal activity of sera to *N. meningitidis* and *N. lactamica*

6.1 Introduction

Complement-dependent bactericidal activity of human serum is considered an important host defence against invasive disease caused by Gram-negative bacteria (Goldschneider *et al.*, 1969a; Rice *et al.*, 1980). Immunity to the meningococcus, both maternally derived and actively induced during life, correlates with the presence of bactericidal activity in normal sera (Goldschneider *et al.*, 1969a; Griffiss *et al.*, 1987b). Adults and older children who become colonized with meningococci develop bactericidal antibody against strains of homologous and heterologous serogroups (Goldschneider *et al.*, 1969a; Reller *et al.*, 1973). This suggests carriage of meningococci plays a role in induction and maintenance of natural meningococcal antibody (Goldschneider *et al.*, 1969a; Reller *et al.*, 1973).

Reller and colleagues (1973) found that colonization of the nasopharynx of adults with non-groupable meningococci which very rarely cause disease can be a potent stimulus for the generation of protection against the capsulate disease causing strains. Among men colonized with serogroup B or C organisms, 87% developed bactericidal antibodies to one or more heterologous strains of the pathogenic meningococci (Goldschneider *et al.*, 1969b). Gotschlich and colleagues (1969a) showed that antibodies to the serogroup A and C polysaccharide were of the IgG, IgM and IgA classes and that IgG antibodies to serogroup A polysaccharide were predominant. It was later reported that the bactericidal activity of acute phase sera from patients infected with serogroup B, C or Y, after removal of IgA, was largely confined to antibodies of the IgM class (Griffiss and Bertram, 1977). Bactericidal antibodies to group B

polysaccharide were mostly of the IgM class (Zollinger and Mandrell 1983; Skevakis *et al.*, 1984).

In this part of the study, the objective was to use a bactericidal assay to assess the following questions with serum specimens from the school children:

1) Is there a difference in serum bactericidal activity against pathogenic and non-pathogenic strains of *Neisseria* ?

2) Is carriage of meningococci or secretor status associated with the bactericidal activity ?

3) Are the levels of different isotypes, either total or specific for *N. lactamica* and *N. meningitidis*, in sera correlated with bactericidal activity?

4) Are there differences in the levels of isotypes, either total or specific for *N. lactamica* and *N. meningitidis*, associated with serum bactericidal activity related to secretor status or carriage?

5) Does the outbreak strain possesses antigens cross-reactive with *N. lactamica* or other meningococcal isolates which might induce bactericidal activity as a result of carriage or systemic infection ?

6.2 Materials and methods

6.2.1 Experimental design

A standard complement source was used throughout the test. This was produced from fresh human AB serum from a single donor. Before use, the complement source was absorbed with a 'mixture' of the meningococcal isolates to be tested by the method described in section 6.2.2 to remove anti-*Neisseria* antibodies. It was standardised in a haemolytic assay to a minimum haemolytic titre of 1/16 to ensure standard amounts of

exogenous complement were present in the test since endogenous complement levels vary between individuals. The absorbed complement source was added (20 µl) to the heat-inactivated test samples. This concentration supplied sufficient complement to mediate antibody-dependent bacteriolysis whilst minimising any bactericidal activity due to activation of the alternative pathway.

Small numbers of bacteria (approximately 10^4 bacteria/ ml) were used in the bactericidal assay to reduce the degree of bacterial clumping and to allow easy determination of the end-point titrations. Serum samples were replaced by GC broth to determine that the observed bacterial killing was not due to activation of the alternative complement pathway. Complement was replaced by GC broth in order to ensure other factors (e.g., residual antibiotics) were not responsible for bactericidal activity. Controls in which complement and serum were replaced by GC broth were also included.

6.2.2 Absorption of serum used as complement source

Serum from a donor of blood group AB with no history of meningococcal or gonococcal infection was absorbed twice over a period of 24 h at 4 °C with a suspension of the *Neisseria* strains (Table 2.1). This was done to remove any reactive antibodies. The bacteria were removed from the serum by centrifugation (1500 g) at 4 °C. The remaining bacteria were removed by filtration, first with a filter with a pore diameter of 5 µm (Nucleopore Corporation, Pleasanton, USA). The absorbed serum was then passed through a sterilising filter with pore diameter of 0.22 µm, Nuflow cellulose acetate membrane (Oxoid Ltd., England). Care was taken to avoid frothing since this might denature the complement proteins. The haemolytic titre of complement was measured as outlined below.

6.2.3 Determination of complement activity

Sheep red blood cells were sensitized with donkey anti-sheep IgG (SAPU). Fresh sheep red blood cells were washed in saline, centrifuged for 5 min. at 300 g and the supernate removed. The wash was repeated 2-3 times. The red blood cells were resuspended in saline to a concentration of 4%. This was checked by centrifugation of the suspension in the haematocrit tube for 5 min. at 300 g. The donkey serum was diluted 1/100 with saline and equal volumes of donkey haemolytic serum and sheep red blood cells were mixed and incubated at 37 °C for 30 min.

Saline (25 µl) was added to one row of U well microtitre plates (Sterilin). The complement source (25 µl) was added to the first well and a series of 7 doubling dilution made. Sensitised sheep red blood cells (25 µl) diluted 1 in 2 were added to each well and the plate incubated at 37 °C for 30 min. The highest dilution that showed complete lysis was recorded as the haemolytic titre. Complement was stored in aliquots at -20 °C until needed.

6.2.4 Assay for bactericidal activity

Sera from 67 secretors and 34 non-secretors were randomly selected from the samples obtained from the school children and heated at 56 °C to inactivate endogenous complement. Each of the 96 wells of an 8 x 12 U well Sterilin microtitre plates was filled with 40 µl D.PBS+B. Heat inactivated neat serum (40 µ) was added to the first well in each column. A series of seven two-fold dilutions was then made and 12 sera were tested per plate.

An eighteen hour culture of the bacteria to be tested was harvested from MNYC medium and suspended in D.PBS+B and the number of bacteria estimated by the O.D of the suspension at 541 nm. The bacteria were diluted to provide a concentration of approximately 10^4 c.f.u./ml. To each well was added 40 µl of one of the 6 bacterial

suspensions. By adding a different bacterial suspension to each plate, the specific bactericidal titres against all six test strains were determined under the same conditions. A 1/16 dilution of the absorbed complement source (20 µl) was added to all the test wells.

Serum and/or complement-free controls were also included in the test. For each strain and each serum specimen (dilution 1/5), controls in which the complement was replaced by GC broth and another in which the serum was replaced by GC broth were included. Controls in which both serum and complement were replaced by GC broth were also included.

The plates were covered and incubated at 37 °C for 30 min. Three 20 µl drops from each well were then plated onto MNYC medium and incubated at 37 °C overnight. The reciprocal of the highest serum dilution at which there was at least an 80% decrease in viable count compared with the controls was recorded as the bactericidal titre.

6.2.5 Absorption of cross-reactive antibodies

Sera from nine individuals were chosen randomly and absorbed with bacteria in two ways.

a) Sera were subjected to a series of absorptions with *N. lactamica* and the outbreak strain (B:4:P1.15). Overnight cultures of bacteria grown on MNYC were harvested by centrifugation (1000 g for 20 min) and washed three times in PBS. Sera were absorbed first with *N. lactamica* by mixing equal volumes of a concentrated suspension of bacteria and sera. Samples were incubated for 60 min at 37 °C followed by incubation overnight at 4 °C. The bacteria were then removed by centrifugation at 1200 g for 5 min with a microcentrifuge (Sorvall MC 12C). After the first absorption, sera were reabsorbed with the outbreak strain using the same method.

b) Each serum sample was divided into five aliquots and absorbed with the following strains: *N. lactamica*, *N. gonorrhoeae* or different strains of *N. meningitidis* (B:4:P1.15, B:15:P1.16 and NG:4:-) by mixing equal volumes of bacteria and serum. Samples were incubated for 60 min at 37 °C followed by incubation overnight at 4 °C. The bacteria were removed by centrifugation at 1200 g for 5 min with a microcentrifuge.

Absorbed and unabsorbed sera were assayed by ELISA for the measurement of the antibody classes (section 4.2.1.2).

6.2.6 Statistical analysis

Immunoglobulin levels of sera with bactericidal activity were compared with those for sera without bactericidal activity by the Mann-Whitney U test. Comparison of the bactericidal titre data was made after conversion of the titres to $\log_2$.

6.3 Results

6.3.1 Enumeration of Bacteria

The optical density of the bacterial suspensions was plotted against the number of colony forming units (c.f.u.) per ml. For each strain, the portion of the curve which approximated a straight line was used to calculate the number of bacteria in the suspension for testing. The best fit line of this section on each one of the graphs was found by linear regression. This is illustrated in Figures 6.1. From the graphs for the individual strains, appropriate dilutions could be made to obtain a suspension containing approximately 10^4 bacteria/ml.

6.3.2 Haemolytic titre of complement

After adsorption of antibodies specific for the test bacteria, the complement source had a haemolytic titre of 1/16.

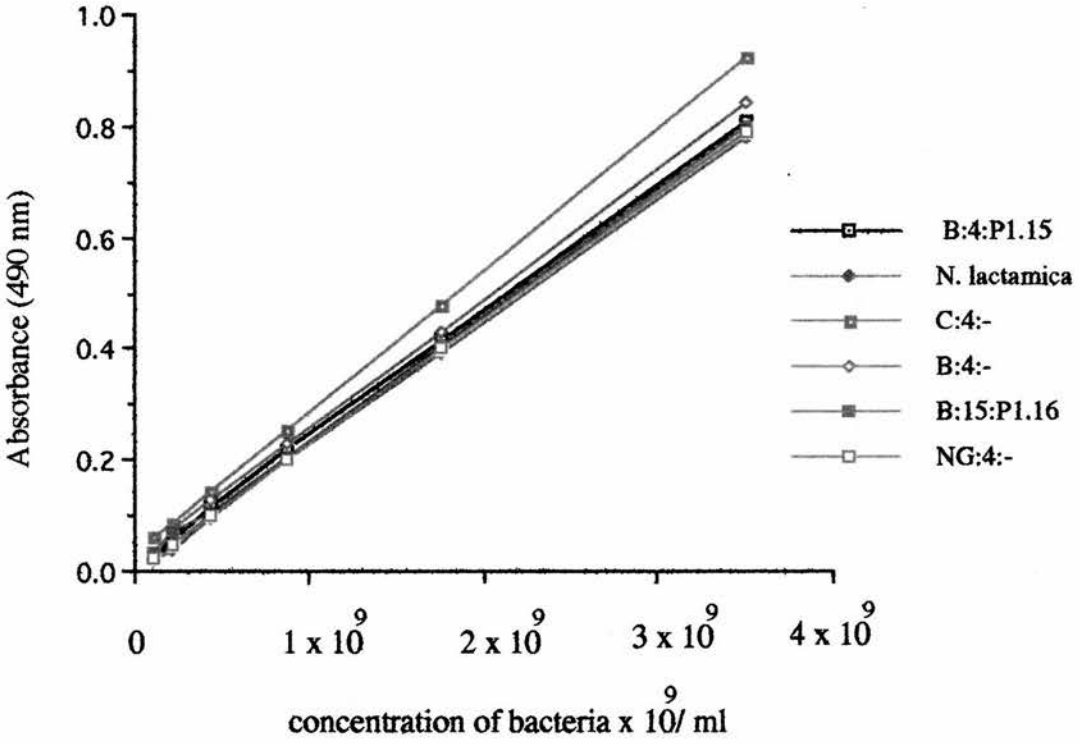

Figure 6.1: Optical density (OD) measurements and bacterial counts determined by viable count (cfu)

6.3.3 Bactericidal antibody activity

Bactericidal antibody titres against *N. lactamica* and different isolates of *N. meningitidis* were measured for 101 individuals. The summary of bactericidal titres of all the sera tested against six strains of *Neisseria* are given in table 6.1. Most of the individuals had a bactericidal titre of at least 128 against NG:4:- (88.9%) and *N. lactamica* (82.4%). The majority of individuals had no bactericidal activity to capsulate strains, B:15:P1.16 (70.4%); C:4:- (72.2%); B:4:- (68.51%).

6.3.4 The effect of secretor status and carriage on bactericidal activity

The effect of carriage on bactericidal activity is shown in Table 6.2. The mean of bactericidal activity on the log scale among non-carriers was lower than that for carriers. Negative titres were assigned a value of zero on the log scale. The slight differences detected were not statistically significant.

Table 6.3 presents the mean of bactericidal titre according to secretor status. Although the mean bactericidal titre on log scale was consistently higher for secretors than non-secretors, the differences were small and not statistically significant.

6.3.5 Correlation between immunoglobulin levels and bactericidal activity

In tables 6.4 - 6.6, the means on the log scale of the levels of total isotype and isotype specific for the panel of bacteria examined in chapter four are correlated with bactericidal activity to the capsulate strains: B:4:P1.15; B:15:P1.16; B:4:-; C:4:-. There was no difference in levels of total IgA or IgM or levels of these isotypes specific for *N. lactamica* or any of the five meningococcal status between sera which showed bactericidal activity compared with sera in which there was no bactericidal activity. Total IgG was significantly higher in sera bactericidal for strains B:4:P1.15 and B:4:-

Table 6.1: Distribution of bactericidal titres against *N. lactamica* and *N. meningitidis* isolates in the population tested.

bactericidal titre	serum killing ≥ 80% of control isolates											
	N. lactamica		B:15:P1.6		C:4:-		B:4:-		B:4:P1.15		NG:4:-	
0*	9	(8.3)	76	(70.4)	78	(72.2)	74	(68.5)	84	(73)	1	(0.9)
2	1	(0.9)	4	(3.7)	4	(3.7)	5	(4.6)	5	(4.3)	1	(0.9)
4	2	(1.9)	7	(6.5)	2	(1.9)	6	(5.6)	3	(2.6)	-‡	
8	2	(1.9)	6	(5.6)	5	(4.6)	6	(5.6)	4	(3.5)	2	(1.9)
16	1	(0.9	3	(2.8)	6	(5.6)	4	(3.7)	1	(0.9)	1	(0.9)
32	1	(0.9)	3	(2.8)	6	(5.6)	4	(3.7)	3	(2.6)	2	(1.9)
64	3	(2.8)	4	(3.7)	3	(2.8)	2	(1.9)	3	(2.6)	5	(4.6)
128	89	(82.4)	5	(4.6)	4	(3.7)	7	(6.5)	12	(10.4)	96	(88.9)

* = No bactericidal activity.

‡ = No bactericidal activity is shown at this titre.

( ) = the percentage of each bactericidal titre.

Table 6.2: Comparison of the means (on $\log_2$ scale) of the bactericidal titres of carriers and non-carriers.

bactericidal titre ($\log_2$)			
strain	carriers n=26	non-carriers n=80	P
N. lactamica	6.19	5.82	0.35
B:4:P1.15	1.49	1.10	0.60
B:15:P1.16	1.49	0.89	0.09
B:4:-	1.86	0.91	0.15
C:4:-	1.19	0.97	0.60
NG:4:-	6.487	6.482	0.99

Table 6.3: Comparison of the means (on $\log_2$ scale) of the bactericidal titres of secretors and non-secretors

bactericidal titre ($\log_2$)			
strain	secretors n=72	non-secretors n=34	P
N. lactamica	5.98	5.78	0.66
B:4:P1.15	1.25	1.08	0.81
B:15:P1.16	1.34	0.90	0.43
B:4:-	1.48	0.98	0.22
C:4:-	1.28	0.94	0.21
NG:4:-	6.52	6.38	0.45

Table 6.4: Comparison of mean (on log scale) of IgA levels between different sera with bactericidal activity

Total and specific IgA	test strain	Bactericidal activity Yes	No	P
		IgA levels µg/ml		
		n=20	**n=55**	
Total *	B:4:P1.15	0.41	0.39	0.28
N. lactamica		0.73	0.88	0.46
B:4:P1.15		0.59	0.74	0.52
B:15:P1.16		0.68	0.85	0.57
B:4:-		0.58	0.90	0.17
C:4:-		0.69	0.84	0.27
NG:4:-		0.82	0.95	0.55
		n=17	**n=58**	
Total	B:15:P1.16	0.42	0.39	0.08
N. lactamica		0.97	0.80	0.44
B:4:P1.15		0.73	0.69	0.74
B:15:P1.16		0.89	0.77	0.35
B:4:-		0.71	0.85	0.68
C:4:-		0.87	0.78	0.67
NG:4:-		0.10	0.88	0.29
		n=17	**n=58**	
Total	B:4:-	0.41	0.39	0.37
N. lactamica		0.77	0.86	0.89
B:4:P1.15		0.71	0.69	0.72
B:15:P1.16		0.76	0.81	0.97
B:4:-		0.81	0.82	0.79
C:4:-		0.91	0.76	0.68
NG:4:-		0.10	0.89	0.38
		n=17	**n=58**	
Total	C:4:-	0.40	0.39	0.78
N. lactamica		0.73	0.88	0.98
B:4:P1.15		0.69	0.70	0.95
B:15:P1.16		0.68	0.85	0.81
B:4:-		0.58	0.90	0.42
C:4:-		0.81	0.80	0.94
NG:4:-		0.83	0.95	0.49

* units for mean total serum IgA are expressed in mg/ml

Table 6.5: Comparison of mean (on log scale) of IgM levels between different sera with bactericidal activity

Total and specific IgM	Test strain	Bactericidal activity Yes	No	P
		IgM (μg/ml)		
		n=21	**n=56**	
Total *	B:4:P1.15	0.41	0.39	0.43
N. lactamica		0.59	0.79	0.15
B:4:P1.15		0.69	0.57	0.35
B:15:P1.16		066	0.91	0.07
B:4:-		1.23	1.15	0.25
C:4:-		0.49	0.62	0.31
NG:4:-		1.01	1.29	0.12
		n=18	**n=59**	
Total	B:15:P1.16	0.41	0.39	0.13
N. lactamica		0.77	0.67	0.65
B:4:P1.15		0.56	0.69	0.39
B:15:P1.16		0.90	0.66	0.10
B:4:-		0.88	1.26	0.25
C:4:-		0.44	0.63	0.24
NG:4:-		1.27	1.03	0.17
		n=17	**n=59**	
Total	B:4:-	0.41	0.39	0.56
N. lactamica		0.75	0.72	0.76
B:4:P1.15		0.58	0.69	0.41
B:15:P1.16		0.75	0.88	0.35
B:4:-		1.22	1.07	0.74
C:4:-		0.62	0.57	0.98
NG:4:-		1.22	1.21	0.55
		n=17	**n=58**	
Total	C:4:-	0.40	0.39	0.29
N. lactamica		0.79	0.67	0.18
B:4:P1.15		0.51	0.70	0.18
B:15:P1.16		0.70	0.89	0.16
B:4:-		1.50	1.10	0.79
C:4:-		0.61	0.66	0.46
NG:4:-		1.26	1.03	0.21

* units for mean total serum IgM are expressed in mg/ml

Table 6.6: Comparison of mean (on log scale) of IgG levels between different sera with bactericidal activity

total and specific IgG	test strain	Bactericidal activity Yes	No	P
		IgG (μg/ml)		
		n=21	**n=56**	
Total *	B:4:P1.15	0.49	0.47	0.03
N. lactamica		1.21	0.56	0.009
B:4:P1.15		0.74	0.62	0.04
B:15:P1.16		0.77	0.57	0.01
B:4:-		0.65	0.44	0.05
C:4:-		0.75	0.59	0.05
NG:4:-		0.81	0.68	0.45
		n=18	**n=59**	
Total	B:15:P1.16	0.49	0.48	0.09
N. lactamica		1.76	0.73	0.05
B:4:P1.15		0.65	0.66	0.57
B:15:P1.16		0.66	0.62	0.83
B:4:-		0.54	0.58	0.76
C:4:-		0.71	0.61	0.13
NG:4:-		0.69	0.72	0.31
		n=18	**n=59**	
Total	B:4:-	0.49	0.47	0.03
N. lactamica		1.24	0.56	0.003
B:4:P1.15		0.75	0.62	0.14
B:15:P1.16		0.70	0.60	0.34
B:4:-		0.55	0.58	0.75
C:4:-		0.72	0.61	0.20
NG:4:-		0.81	0.68	0.33
		n=18	**n=59**	
Total	C:4:-	0.48	0.47	0.22
N. lactamica		1.19	0.59	0.01
B:4:P1.15		0.72	0.63	0.14
B:15:P1.16		0.71	0.59	0.32
B:4:-		0.60	0.56	0.47
C:4:-		0.75	0.59	0.16
NG:4:-		0.82	0.69	0.83

* units for mean total serum IgG are expressed in mg/ml

compared with sera in which there was no bactericidal activity. IgG levels for *N. lactamica* were significantly higher in sera bactericidal for all four strains tested compared with sera in which there was no bactericidal activity. IgG levels for B:15:P1.16, B:4:- and C:4:- were significantly higher in sera bactericidal for the outbreak strain compared with sera in which there was no bactericidal activity, but not for any of the other three strains tested. Compared with sera in which there was no bactericidal activity, there were no differences in levels of IgG for NG:4:- in sera bactericidal for any of the four strains.

6.3.6 Correlation between IgG levels and bactericidal activity among carriers and non-carriers

Tables 6.7 and 6.8 present similar data for assessment of differences in total and specific IgG levels and bactericidal activity with reference to carriage. Among carriers, compared with sera in which there was no bactericidal activity, there was no differences in the amount of total IgG; however, there were significantly higher levels of IgG specific for *N. lactamica* in sera with bactericidal activity for each of the four capsulate strains. There were higher levels of IgG specific for B:4:P1.15 in sera bactericidal for B:15:P1.16 but not for the outbreak strain itself or B:4:- and C:4:- compared with sera in which there was no bactericidal activity. Higher levels of IgG specific for B:15:P1.16 or B:4:- were not found in sera bactericidal for any of the four strains tested compared with sera in which there was no bactericidal activity. Significantly higher levels of IgG to C:4:- were found only among sera bactericidal for B:15:P1.16 compared with sera in which there was no bactericidal activity. No difference in the levels of IgG to NG:4:- were associated with bactericidal activity to any of the strains (Table 6.7).

Among non-carriers, compared with sera in which there was no bactericidal activity, the only significant increases in levels of IgG associated with bactericidal activity were those for *N. lactamica* and killing of B:4:P1.15 or B:4:- (Table 6.8).

Table 6.7: Mean of IgG levels (on log scale) in sera with bactericidal activity compared with non-bactericidal sera obtained from carriers

Total and specifc IgG	Test strain	Bactericidal activity Yes	No	P
		IgG (µg/ml)		
		n=4	**n=10**	
Total *	B:4:P1.15	0.49	0.47	0.06
N. lactamica		2.43	0.53	0.02
B:4:P1.15		0.77	0.63	0.19
B:15:P1.16		0.88	0.52	0.09
B:4:-		0.72	0.68	0.20
C:4:-		0.80	0.65	0.25
NG:4:-		0.88	0.67	0.55
		n=4	**n=10**	
Total	B:15:P1.16	0.49	0.48	0.36
N. lactamica		1.40	0.71	0.05
B:4:P1.15		0.89	0.63	0.03
B:15:P1.16		0.89	0.55	0.16
B:4:-		0.83	0.55	0.09
C:4:-		0.94	0.61	0.005
NG:4:-		0.92	0.68	0.57
		n=5	**n=9**	
Total	B:4:-	0.49	0.48	0.39
N. lactamica		2.37	0.56	0.004
B:4:P1.15		0.71	0.68	1.00
B:15:P1.16		0.72	0.61	0.74
B:4:-		0.65	0.63	0.74
C:4:-		0.71	0.70	0.84
NG:4:-		0.87	0.67	0.74
		n=4	**n=10**	
Total	C:4:-	0.49	0.48	0.48
N. lactamica		2.87	0.53	0.003
B:4:P1.15		0.73	0.65	0.19
B:15:P1.16		0.76	0.60	1.00
B:4:-		0.58	0.66	0.67
C:4:-		0.72	0.69	1.00
NG:4:-		0.92	0.68	0.57

* units for mean total serum IgG are expressed in mg/ml

Table 6.8: Mean of IgG levels (on log scale) in sera with bactericidal activity compared with non-bactericidal sera obtained from non-carriers.

Total and specific IgG	Test strain	Bactericidal activity Yes	No	P
		IgG (µg/ml)		
		n=16	**n=47**	
Total *	B:4:P1.15	0.49	0.47	0.13
N. lactamica		0.82	0.57	0.02
B:4:P1.15		0.72	0.62	0.13
B:15:P1.16		0.73	0.85	0.09
B:4:-		0.62	0.54	0.29
C:4:-		0.73	0.57	0.15
NG:4:-		0.79	0.68	0.63
		n=17	**n=47**	
Total	B:15:P1.16	0.49	0.47	0.14
N. lactamica		0.78	0.59	0.09
B:4:P1.15		0.61	0.65	1.00
B:15:P1.16		0.58	0.63	0.52
B:4:-		0.46	0.59	0.22
C:4:-		0.64	0.61	0.72
NG:4:-		0.62	0.74	0.12
		n=15	**n=48**	
Total	B:4:-	0.49	0.47	0.06
N. lactamica		0.86	0.56	0.003
B:4:P1.15		0.77	0.61	0.13
B:15:P1.16		0.69	0.59	0.32
B:4:-		0.51	0.57	0.57
C:4:-		0.72	0.59	0.16
NG:4:-		0.66	0.73	0.16
		n=14	**n=49**	
Total	C:4:-	0.48	0.47	0.29
N. lactamica		0.72	0.61	0.18
B:4:P1.15		0.70	0.60	0.19
B:15:P1.16		0.69	0.59	0.36
B:4:-		0.61	0.54	0.29
C:4:-		0.76	0.58	0.13
NG:4:-		0.78	0.69	0.86

* units for mean total serum IgG are expressed in mg/ml

6.3.7 Correlation between IgG levels and bactericidal activity among secretors and non-nonsecretors

Secretors had significantly higher levels of IgG antibodies to *N. lactamica* in the sera which contained bactericidal activity compared with sera in which there was no bactericidal activity. The only other significant association was higher levels of IgG specific for C:4 in sera bactericidal for that strain (Table 6.9). There were no significant differences in IgG levels to *N. lactamica* among non-secretors sera with bactericidal activity compared with sera in which there was no bactericidal activity. Sera of non-secretors that showed bactericidal activity to the outbreak strain had higher levels of IgG specific for B:15:P1.16 (P=0.01) and B:4:- (P=0.03) compared with sera in which there was no bactericidal activity (Table 6.10).

6.3.8 Comparison between secretor and non-secretor sera with bactericidal activity

Although secretors had higher IgG levels to *N. lactamica* in the sera which had bactericidal activity compared with non-secretors, the differences were not statistically significant (Table 6.11).

6.3.9 Cross-reactivity among strains of *Neisseria*

The presence of antibodies to B:4:P1.15 in serum of randomly selected specimens was demonstrated by the ELISA (Tables 6.12 and 6.13).

Serum IgA, IgG and IgM antibodies to the strain were measured by ELISA before and after absorptions with *N. lactamica*, *N. gonorrhoeae* or different isolates of *N. meningitidis* . The reduction in measurable serum IgA, IgG and IgM in the absorbed serum indicated that there are substantial amounts of antibodies in sera cross-reactive with the outbreak strain (Table 6.12).

Table 6.9: Mean of IgG levels (on log scale) in sera with bactericidal activity compared with non-bactericidal sera from secretors.

Total and specific IgG	Test strain	Bactericidal activity Yes	No	P
		IgG (µg/ml)		
		n=15	**n=40**	
Total*	B:4:P1.15	0.48	0.47	0.12
N. lactamica		1.45	0.61	0.01
B:4:P1.15		0.73	0.66	0.26
B:15:P1.16		0.76	0.59	0.16
B:4:-		0.59	0.54	0.59
C:4:-		0.76	0.61	0.21
NG:4:-		0.83	0.66	0.38
		n=12	**n=43**	
Total	B:15:P1.16	0.48	0.47	0.25
N. lactamica		1.69	0.62	0.005
B:4:P1.15		0.59	0.70	0.53
B:15:P1.16		0.66	0.62	0.89
B:4:-		0.49	0.57	0.44
C:4:-		0.73	0.63	0.25
NG:4:-		0.74	0.70	0.88
		n=12	**n=43**	
Total	B:4:-	0.48	0.47	0.22
N. lactamica		1.72	0.59	0.0001
B:4:P1.15		0.80	0.64	0.27
B:15:P1.16		0.70	0.61	0.81
B:4:-		0.50	0.57	0.65
C:4:-		0.77	0.62	0.22
NG:4:-		0.80	0.68	0.81
		n=10	**n=45**	
Total	C:4:-	0.48	0.47	0.41
N. lactamica		1.71	0.65	0.05
B:4:P1.15		0.76	0.66	0.18
B:15:P1.16		0.79	0.59	0.21
B:4:-		0.60	0.55	0.46
C:4:-		0.89	0.59	0.003
NG:4:-		0.97	0.65	0.22

* units for mean total serum IgG are expressed in mg/ml

Table 6.10: Mean of IgG levels (on log scale) in sera with bactericidal activity compared with non-bactericidal sera obtained from non-secretors.

Total and specif IgG	Test strain	Bactericidal activity Yes	No	P
		IgG (µg/ml)		
		n=6	**n=16**	
Total *	B:4:P1.15	0.50	0.47	0.16
N. lactamica		0.60	0.45	0.23
B:4:P1.15		0.74	0.54	0.57
B:15:P1.16		0.79	0.53	0.01
B:4:-		0.78	0.55	0.03
C:4:-		0.72	0.53	0.07
NG:4:-		0.74	0.73	0.88
		n=6	**n=16**	
Total	B:15:P1.16	0.49	0.47	0.33
N. lactamica		0.93	0.90	0.76
B:4:P1.15		0.77	0.53	0.57
B:15:P1.16		0.63	0.59	0.61
B:4:-		0.64	0.56	0.55
C:4:-		0.65	0.60	0.18
NG:4:-		0.58	0.79	0.08
		n=8	**n=14**	
Total	B:4:-	0.49	0.47	0.22
N. lactamica		0.51	0.48	0.63
B:4:P1.15		0.69	0.53	0.57
B:15:P1.16		0.69	0.55	0.17
B:4:-		0.62	0.61	0.50
C:4:-		0.63	0.56	0.22
NG:4:-		0.59	0.82	0.81
		n=6	**n=16**	
Total	C:4:-	0.49	0.47	0.61
N. lactamica		0.57	0.45	0.24
B:4:P1.15		0.68	0.53	0.43
B:15:P1.16		0.61	0.59	0.89
B:4:-		0.60	0.62	0.89
C:4:-		0.57	0.59	0.89
NG:4:-		0.62	0.80	0.08

* units for mean total serum IgA are expressed in mg/ml

Table 6.11: Comparison of mean (on log scale) of IgG levels to *N. lactamica* between secretors and non-secretors of sera with bactericidal activity

test strain	secretors	non-secretors	p
	IgG levels µg/ml		
	n=55	**n=20**	
B:4:P1.15	14.5	6.0	0.28
	n=58	**n=17**	
B:15:P1.16	16.9	9.3	0.08
	n=58	**n=17**	
B:4:-	17.2	5.1	0.38
	n=58	**n=17**	
C:4:-	17.1	5.7	0.78

Table 6.12: The level of antibodies to B:4:P1.15 detected by whole cell ELISA before and after absorption with different strains of *Neisseria*

Serum		Unabsorbed serum µg/ml	% of reduction compared with unadsorbed sera Absorbed with				
			B:4:P1.15	*N. lactamica*	*N. gonorrhoae*	B:15:P1.16	NG:4:-
1	IgA	8.3	70*	57	22	47	63
	IgM	9.7	94	83	31	74	86
	IgG	23.0	88	51	38	38	56
2	IgA	15.6	90	60	47	47	62
	IgM	11.5	85	67	32	50	62
	IgG	25.0	78	50	46	53	64
3	IgA	7.3	85	43	15	35	46
	IgM	8.0	90	87	65	74	80
	IgG	17.0	97	73	47	56	74
4	IgA	12.7	71	51	37	42	45
	IgM	8.03	92	46	32	41	53
	IgG	30.0	98	76	25	46	76
5	IgA	15.8	84	30	11	15	54
	IgM	7.2	95	65	34	33	44
	IgG	32.0	95	76	38	28	73
6	IgA	8.40	85	64	35	30	61
	IgM	5.50	81	68	28	47	55
	IgG	11.0	96	48	12	43	57
7	IgA	2.97	85	33	12	13	38
	IgM	1.70	79	60	19	39	53
	IgG	7.00	90	59	35	59	58
8	IgA	6.88	57	35	17	26	37
	IgM	4.40	68	33	14	19	43
	IgG	18.3	94	75	19	40	63
9	IgA	9.38	76	47	27	34	37
	IgM	4.40	79	52	18	36	40
	IgG	20.3	82	59	11	37	55

* the percentage of reduction.

Table 6.13: The level of antibodies to B:4:P1.15 detected by whole cell ELISA before and after absorption with B:4:P1.15 and/or *N. lactamica*

Serum		Unabsorbed serum µg/ml	% reduction compared with unadsorbed sera Absorbed with		
			B:4:P1.15	*N. lactamica*	B:4:P1.14 + *N. lactamica*
1	IgA	8.3	70*	57	72
	IgM	9.7	94	83	94
	IgG	23.0	88	51	88
2	IgA	15.6	90	60	90
	IgM	11.5	85	67	89
	IgG	25.0	78	50	79
3	IgA	7.3	85	43	86
	IgM	8.0	90	87	90
	IgG	17.0	97	73	98
4	IgA	12.7	71	51	85
	IgM	8.03	92	46	93
	IgG	30.0	98	76	98
5	IgA	15.8	84	30	84
	IgM	7.2	95	65	95
	IgG	32.0	95	76	96
6	IgA	8.40	85	64	87
	IgM	5.50	81	68	84
	IgG	11.0	96	48	96
7	IgA	2.97	85	33	85
	IgM	1.70	79	60	81
	IgG	7.00	90	59	92
8	IgA	6.88	57	35	61
	IgM	4.40	68	33	70
	IgG	18.3	94	75	96
9	IgA	9.38	76	47	77
	IgM	4.40	79	52	79
	IgG	20.3	82	59	83

* the percentage of reduction.

Given the heterogenecity of the antibody response, a pronounced antibody reduction was observed when the sera were absorbed twice, first with *N. lactamica* followed by B:4:P1.15. A reduction of IgG antibodies ranging from 79% to 98% was observed after the sera were absorbed in this manner (Table 6.13).

6.4 Discussion

The results obtained provided data to answer the questions posed in the introduction. (1) Most sera in this study had higher bactericidal titres against the usually non-pathogenic strains NG:4:- (88.9%) and *N. lactamica* (82.4%) compared to the capsulate strains. (2) Bactericidal activity was higher for both carriers and secretors but the differences were not significant. (3) Compared with sera in which there was no bactericidal activity, in bactericidal sera there was no marked difference in levels of total or specific IgA and IgM. IgG levels were significantly higher in sera bactericidal for all four strains tested compared with sera in which there was no bactericidal activity. (4) Carriers had significantly higher levels of IgG to *N. lactamica* in sera with bactericidal activity for each of the four capsulate strains compared with sera in which there was no bactericidal activity. While there were higher levels of IgG to *N. lactamica* in sera of non-carriers with bactericidal activity, this was significantly associated only with killing of B:4:P1.15 and B:4:-. Secretors' sera with bactericidal activity had significantly higher levels of IgG to *N. lactamica* compared with sera that were not bactericidal. There was no significant association of IgG levels and bactericidal activity to *N. lactamica* among non-secretors. (5) Antibodies to the outbreak strain B:4:P1.15 could be adsorbed by all *Neisseria* isolates tested.

Many previous studies of the serum bactericidal reaction with meningococci have focused on the specificity and chemical composition of the target antigens (Goldschneider *et al.*, 1969b; Kasper *et al.*, 1973b). Others have focused on the classes of immunoglobulin involved in bactericidal killing by using antibodies from

human sera obtained after infection with meningococci (Griffiss *et al.*, 1975) or sera collected after vaccination (Skevakis *et al.*, 1984). In this study, levels of isotypes to the outbreak strain and other *Neisseria* species in sera obtained from a school population in which an outbreak of meningococcal disease occurred were measured.

It has been suggested that carriage of commensal enteric bacteria with cross-reactive antigens might induce IgA antibodies that compete with IgG and IgM (Griffiss *et al.*, 1975). The results presented here suggest that the effect of circulating IgA antibodies is not important. There was no statistical difference in levels of IgA between the sera with bactericidal activity compared with sera in which no killing was observed. The level of IgA might not be sufficient to block IgG and IgM in the sera tested for bactericidal activity. This is in agreement with Griffiss and colleagues (1975) who found that strain specific IgA isolated from convalescent-phase sera was capable of blocking the complement-mediated bacteriolytic activity of IgM and IgG separated from the same sera; however, the blocking effect of IgA was not demonstrable when using whole sera.

The results of the present study showed that the IgM levels of bactericidal and non-bactericidal sera were comparable. This might be due to the fact that there were about 15 days between the first screening of the school and treatment with rifampicin and the the second screening when sera were collected. The levels of IgM might have decreased, or there was no significant difference in the level of IgM between the groups.

Previous workers have studied the IgG subclasses and found that the profile of IgG subclass in the immune response varies according to the antigenic stimulus and might reflect the mechanism and efficacy by which the meningococci are killed (Natvig and Kunkel, 1973; Burton *et al.*, 1986). Protein antigens mainly stimulate the production of IgG1 and IgG3 antibodies (Hammarstrom and Smith, 1986). Sjursen and

colleagues (1990) found that the antibody levels in vaccinees and patients' sera were comparable. Both had high levels of IgG with IgG1 and IgG3 being demonstrated in both groups. They also found that the level of the total IgG and IgG1 remained high for over three years which suggested that this might be stimulated by repeated exposure to meningococci or cross reactive antigens on other species.

The higher levels of the IgG to *N. lactamica* in the sera with bactericidal activity to all the capsular strains used, regardless of serogroups and serotypes suggests that these antibodies might be directed to common antigens. This agrees with the suggestion that noncapsular surface antigens shared between different meningococci play a role in the induction and maintenance of natural meningococcal antibody in the population (Goldschneider *et al.*, 1969a). Sera from healthy individuals usually contain IgG antibodies against outer membrane antigens from pathogenic *Neisseria* species (Harthug *et al.*, 1986; Hicks *et al.*, 1987).

Commensal *Neisseria* species *N. lactamica* and *N. cinerea* are closely related to the pathogenic strains on the basis of a variety of criteria (Aho *et al.*, 1987). *N. meningitidis* and *N. lactamica* share outer membrane proteins. These strains synthesize the H.8 lipoprotein antigen (Aho *et al.*, 1987; 1984; Kawula *et al.*, 1987). Antibodies against the H.8 protein are found in patients after disseminated meningococcal and gonococcal disease (Black *et al.*, 1985). Martin and colleagues (1986) described a 70 kilodalton protein that is present in both pathogenic and most non-pathogenic species, including *N. lactamica.* The importance of each of the various common *Neisseria* antigens as a target for protective antibodies still remains to be clarified.

N. lactamica is one of the first *Neisseria* species to colonize the infant's pharynx. By four years of age 59% of children have carried *N. lactamica* at least once (Gold *et al.*, 1978a). They observed that 66% of the children (3 months to 8 years of age) who

became colonized with *N. lactamica* developed significant levels of serum antibodies which can bind meningococcal test strains of serogroups A, B and/or C; and 40% developed bactericidal antibody against these strains.

In contrast to the serogroupable strains examined, most of the sera were bactericidal for *N. lactamica* and NG:4:-. This suggests that these two strains are more sensitive to the bactericidal activity of acquired antibodies than serogroupable isolate. Rosenqvist and colleagues (1988) found that IgG antibody levels were significantly higher for carriers than for non-carriers before and after vaccination.

The proposal for a role of the carrier state in developing and maintaining natural immunity to meningococcal disease has been supported by other workers (Goldschneider *et al.*, 1969a; Reller *et al.*, 1973). In this study of a non-vaccinated population, the patterns of reactivity of antibodies in sera from carriers and non-carriers to the four capsulate isolates are different. Generally among non-carriers, there was no significant association between bactericidal activity and the presence of IgG antibodies to these strains. In contrast, among carriers, sera bactericidal for the capsulate isolates tested had high levels of the IgG to *N. lactamica.*

Nongroupable strains of *N. meningitidis* are usually isolated from the throat and rarely cause disease (Meningococcal Disease Surveillance Group, 1976b). These isolates probably have either uncharacterized polysaccharide capsule or easily lack detectable amount of capsular polysaccharide. The majority of individuals (99.1%) showed bactericidal activity to NG:4:- compared with little bactericidal activity against encapsulated strains sharing the same serotype antigen (Table 6.1). One possible explanation is that the heavily encapsulated strains might impede the functional activity of antibodies to the underlying proteins or lipopolysaccharide. These observations need to be considered in assessment of serotype antigens for their use as vaccines for serogroupable meningococci.

Compared to non-secretors, secretors had higher IgG levels to *N. lactamica* in sera showing bactericidal activity for all four isolates tested. The differences were not significant, which is in agreement with the results obtained in chapter four when the whole population were tested for levels of total and specific IgG.

By means of the absorption experiments, antigenic similarities have been demonstrated among different strains of *Neisseria.* Antibody levels were measured by ELISA to the outbreak strain and *N. lactamica* before and after absorption with homologous and heterologous isolates. Immunoglobulin levels in the serum were reduced by various degrees after absorption with different strains of *Neisseria* in the nine sera tested. Absorption of each serum sample with *N. lactamica* followed by another absorption with the outbreak strain reduced the level of IgG antibodies to the outbreak strain by 73 - 93%. Reduction of IgA and IgM antibodies was also evident. Identification of the bacterial determinants to which these antibodies are directed might be useful in assessment of vaccine candidates.

General discussion

The results have been discussed in detail at the end of each chapter. This chapter summarizes the findings and conclusions with reference to the original objectives set out in the general introduction, explores the limitations of the experimental methods used and the prospects for future research.

7.1 Introduction

Non-secretors of blood group antigens are over-represented among patients with bacterial meningitis. The study presented here tested whether there might be differences between secretors and non-secretors in terms of their mucosal or systemic immune response. To test the hypothesis, serum and salivary immunoglobulin levels were measured and the functions of the immunoglobulin were studied by assessment of bactericidal activity and the effect of salivary antibodies on bacterial binding to epithelial cells.

7.2 The association between the immunoglobulin levels and secretor status.

The first part of the study was designed to assess the reported lower levels of serum and salivary antibodies reported for non-secretors. An ELISA was developed to assess the level of total and specific antibodies in serum and saliva. Although there are several methods suitable for the measurement of total immunoglobulins, ELISA was chosen for this study because it was a more sensitive technique compared with SRID, the other method usually used for this purpose. In addition, specific antibodies can be determined and a large number of samples can be analyzed quickly. The ELISA technique developed in this study can be applied to quantitative examination of immune responses to other antigens for which there are no standard sera available.

Non-secretors had significantly less total IgM in their saliva compared with secretors. These differences were also observed for salivary IgM to *N. lactamica* and to five isolates of meningococci expressing different combinations of serogroup, serotyp and subtype antigens. As there was no correlation between salivary and serum levels of IgM, the results suggest that salivary IgM is loccally produced and has not leaked from the serum.

Previous studies on the association between secretor status ans immunoglobulin levels failed to analyse several of the factors that might influence the results. In this study 39 variables were analysed including: secretor status; carriage of meningococci; smoking habits; total serum and salivary IgA, IgG and IgM. In addition, specific salivary IgA and IgM, and serum IgA, IgG and IgM to six different isolates of *Neisseria* were assessed. The advantage of multivariant analysis is that it takes into account factors that might be dependent variables which could obscure the results of univariant analysis. Most other studies failed to look at all the factors outlined above.

The lower level of IgM in non-secretors might contribute to susceptibility to colonization, particularly among infants under the age of 12 months in whom salivary IgM is the major class of antibodies on the mucosal surfaces. In early childhood IgM, might compensate for the relative immaturity of the secretory IgA response. Neonatal B lymphocytes have been shown to be functionally defective in their capacity to generate plasma cells, and this immaturity is reflected particularly in the IgA response (Miyawaki *et al.*, 1981). Healthy neonates are able to produce sIgA antibodies (Lodinova-Zadinkova *et al.*, 1991), but the levels remain at the lower end of the adult normal range until the age of 12 months (Mellander *et al.*, 1984). These studies need to be repeated with material from infants who have been screened for carriage of commensal *Neisseria* and meningococci.

7.3 Inhibition of meningococcal attachment to epithelial cells

Despite the importance of the initial colonization of mucosal surfaces by pathogenic micro-organisms, very little is known about the molecules interacting in the process of attachment. A major problem in studying the pathogenesis of meningococci is the specificity of the disease for humans. This limits the use of animal models. To overcome this problem, the attachment of meningococci to epithelial cells was studied *in vitro* to test inhibitory activity of whole saliva and purified sIgA and sIgM. A flow cytometry technique was developed to assess the attachment of meningococci to epithelial cells under the conditions examined.

Whole saliva was used first to study its inhibitory activity. Treatment of bacteria with saliva inhibited the attachment of meningococci. When the saliva was absorbed with a cocktail of meningococci or absorbed then filtered, the inhibitory activity of these saliva preparations was considerably less than that of untreated saliva; however, the inhibitory activity was not due solely to antibodies to the bacteria. Compared with specimens from non-secretors, secretors had significantly higher levels of inhibitory activity in either the fresh saliva from individual donors or pooled saliva. The difference between the two groups raised the possibility that sIgM and/or other factors such as secreted blood group antigens played a role in inhibition of bacterial binding. There is evidence that the Lewis^a antigen usually found in greater quantities on cells and in body fluids of non-secretors is one of the receptors for attachment for some strains of *Staphylococcus aureus* (Blackwell *et al.*, 1992b; Saadi *et al.*, 1993) and *Candida albicans* (May *et al.*, 1989; Tosh and Douglas, 1991; Aly, 1992). The role of this antigen in colonization by meningococci needs to be examined, especially as it is expressed in significant quantities in most infants (Issit, 1986) and by a significant number of secretors (Blackwell *et al.*, 1992b; Saadi *et al.*, 1993). Outer membrane proteins from both *S. aureus* and *N. meningitidis* have been obtained by affinity purification with synsorb Le^a (Saadi *et al.*, submitted for publication; Raza *et al.*, unpublished results); and >

75% of meningococcal strains were agglutinated by anti-idiotypic antibodies produced by immunization of mice with monoclonal anti-Le^a (Essery *et al.*, submitted for publication).

Affinity purified preparations of IgA and IgM antibodies were tested for their ability to inhibit attachment of meningococci. The results, though statistically significant, did not indicate a high level of inhibition. The results do not conclusively indicate a role for purified sIgA or sIgM in the inhibition of attachment of meningococci. This might be due to low amounts of purified sIgA specific for bacterial adhesins, the process of purification affected the sensitivity of IgA, or IgA functions better in cooperation with other factors in the saliva. A panel of saliva from which either IgG or IgM had been absorbed demonstrated higher levels of inhibition compared with purified antibodies, suggesting that purified antibodies played a minor part in the inhibitory activity observed.

The integrity of mucosal defences in many IgA-deficient individuals demonstrates the effectiveness of innate factors or sIgM. During the maturation of the infant's own immune system, the mucosal surfaces are protected by non-immune factors (Tenovuo *et al.*, 1986) and immune factors such IgA in breast milk. In addition, sIgM might play an important role in protection since its concentration is high during this period. It is probably naive to consider any single defence factor in isolation because successful mucosal protection is clearly due to a number of interactions between innate and specific immune defences.

The conflicting results of the role of sIgA in inhibition of attachment of bacteria might be explained in many ways, including differences in populations under study, sample collections, assay methods used and antibodies which might bind to a variable degree to cross-reactive antigens on oral bacteria.

Buccal epithelial cells used in this study are not the normal site of attachment of meningococci, but the cells are easily available and have been used in many studies (Craven *et al.*, 1980; Salit and Morton, 1981; Stephens, 1989). The binding assay might differ with different samples of buccal epithelial cells due to the presence of molecules of food debris, normal flora, inherited differences between individuals, the degree of maturity or viability of the cells. Pharyngeal cells can also be obtained relatively easily; but, their use *in vitro* bacterial attachment assays is limited by similar factors. The use of cell lines such as HeLa provide a homogeneous population free of contaminantes; however, cell surface characteristics might be different from those expressed by cells *in vivo* .

The importance of pili in meningococcal adhesion cannot be ignored. Pili are the most important surface components mediating initial attachment of meningococci to human mucosal epithelial cells (Stephens and Whitney, 1985). Genital secretions obtained after gonococcal infection and after administration of a vaccine containing components to pili have IgG and IgA antibodies which decreased adherence of homologous gonococcal strains (Tramont *et al.*, 1980; McChesney *et al.*, 1982). Stephens and McGee (1981) demonstrated that non-pilate meningococci bound at low levels with equal ability to different cells. In contrast, the presence of pili, in addition to enhancing attachment, also allowed meningococci to bind with differing affinities to epithelial cells from different sites.

One major criticism of binding assays with flow cytometry is that the influence of pili cannot be studied because of the washing step needed to remove unbound fluorescein from the bacterial preparation. The other adhesive mediators used by the bacteria could still be investigated by the assay. Extensive immunologic diversity of pili has been reported (Olafson *et al.*, 1985; Heckels, 1989). The diversity is present even among pili isolated from the same strain and is a major problem in the design of vaccines that contain pili as essential constituents. It is yet to be shown that vaccines containing pili

can produce significant amounts of protective antibody at mucosal surfaces for prolonged periods of time.

7.4 Bactericidal activity of sera from secretors and non-secretors

Sera from the school children were screened for bactericidal antibodies against isolates of *Neisseria*. Most of the individuals had high titres of antibodies to *N. lactamica* and *N. meningitidis* NG:4:- but not to capsulate isolates. Since antigens of *N. lactamica* are cross-reactive with other neisseria and they do not express capsules, the results were expected to be similar to those for the NG:4:- strain.

Compared with non-carriers, carriers had high levels of IgG to *N. lactamica* and it was associated with bacteriacidal activity to the encapsulated isolates examined. Carriage might boost production of antibody to common epitopes and induce strain or species specific antibody production. Capsular polysaccharides do not appear to contribute significantly to cross-reactive antibodies as sera bactericidal for the serogroup B isolates tested were usually bactericidal for serogroup C.

One of the major factors responsible for differences in studies of bactericidal assays might be the sources of complement. The majority used sera obtained from rabbits, and McCutchan and colleagues (1978) observed that gonococcal strains resistant to normal human serum are killed by normal rabbit serum. It is, therefore, essential to use human serum to get reliable results for humans.

There are wide variations in the ways the results of bactericidal activity are reported, generally as percentages of death or survival of bacteria. Many investigators consider significant killing of bacteria has occurred if there is a 50% reduction in viable bacteria compared with the control (Skevakis *et al.*, 1984; Rosenqvist *et al.*, 1988). The present study used 80% reduction of viable bacteria as significant killing which reduces the probability that the results were due to experimental error.

The increased susceptibility of non-secretors to meningococcal disease compared with secretors might be partly explained by results of this this study. First, non-secretors had significantly lower level of total IgM and IgM to *N. lactamica* and five isolates of *N. meningitidis* in saliva. These differences might be important in infants in whom IgM is the major class of antibodies on the mucosal surfaces. Second, the susceptibility could be due to lower levels of inhibition of binding to epithelial cells with saliva obtained from non-secretors. This might be explained either by the lower levels of sIgM in non-secretors or by differences in innate defences (inhibitory glycoconjugates, lactoferrin, lysozyme and lactoperoxidase) of non-secretors compared with secretors. The latter possibility needs to be investigated as absorption of anti-meningococcal antibodies did not completely remove the inhibitory activity. Third, secretors' sera had higher levels of IgG antibodies to *N. lactamica,* a factor that has been associated with bactericidal activity to encapsulated isolates. The differences, however, were not statistically significant. Further studies need to examine IgG subclasses and particularly opsonic activities of sera using the flow cytometry technique.

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Secretor status and humoral immune responses to *Neisseria lactamica* and *Neisseria meningitidis*

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SUMMARY

Non-secretors of ABO blood group antigens are over-represented among patients with meningococcal diseases. Lower levels of secretory IgA reported for non-secretors have been suggested to compromise mucosal defences. Total serum and salivary IgG, IgA and IgM and levels of these isotypes specific for *Neisseria lactamica* and five isolates of meningococci were determined by ELISA for 357 pupils and staff of a secondary school in which an outbreak of meningitis occurred. There were no differences in total or specific levels of serum IgG, IgA or IgM or salivary IgG or IgA of secretors compared with non-secretors. Non-secretors had significantly lower levels of salivary IgM ($P = 0{\cdot}022$) A similar pattern was observed for levels of IgM specific for *N. lactamica* and five meningococcal isolates. The significance of these results is discussed with reference to the role of secretory IgM in protection of mucosal surfaces in infants.

INTRODUCTION

The ability of the host to resist infection or colonization by microorganisms is partly dependent on the presence of a fully functional mucosal immune system. Although IgA is the most abundant immunoglobulin in exocrine secretions such as tears, saliva and milk, IgG and IgM are also found in these fluids [1, 2]. IgM can also function as a true secretory immunoglobulin [3], reaching the mucosal surface by the identical secretory-component mediated transcellular pathway that transports IgA. The full protective potential of secretory IgA is not present in the infant at birth [4, 5]. Adult levels of secretory IgA are not reached until after 1 year of age [6, 7]. It has been suggested that in infants the presence of IgM compensates partially for the low levels of IgA [7]. IgM to poliovirus and *Escherichia coli* has been found in infants [7, 8].

Protective immunity to disease due to *Neisseria meningitidis* is associated with the presence of an intact complement system and opsonizing or bactericidal antibodies specific for the invading strain [9–11]. These antibodies can be formed through nasopharyngeal carriage of meningococci [12]. The majority of individuals

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who develop invasive disease lack protective antibody to the pathogen [9, 10]. The age range in which meningococcal infection is most prevalent (6 months to 4·5 years) reflects the natural development of antibodies to these pathogens.

Non-secretors of ABO blood group antigens are over-represented among patients with disease due to *N. meningitidis* [13, 14] and among carriers of this bacterium [15]. The lower immunoglobulin levels found in non-secretors compared with secretors have been used to explain the increased susceptibility of non-secretors to rheumatic fever and rheumatic heart disease [16, 17]. In later studies, non-secretors were reported to have lower levels of both serum [18] and salivary [19] IgA. It was suggested that specific immune responses at the mucosal surfaces of non-secretors might be compromised compared with that of secretors. The aims of the present study were to determine:

(1) if there is a difference in the amount of total serum and salivary IgA, IgG and IgM between secretors and non-secretors;
(2) if there is a difference in the levels of isotypes specific for *N. lactamica* and *N. meningitidis* in serum or saliva of secretors and non-secretors;
(3) if there is a correlation between levels of specific anti-meningococcal antibodies in secretions and in serum;
(4) if carriage of meningococci affects the levels of antibody to these bacteria found in secretors and non-secretors.

MATERIALS AND METHODS

Subjects

Sera and whole saliva specimens (357) were obtained from staff and pupils (most within the 12–18 years age group) of a school in which there was an outbreak of meningococcal disease due to a serogroup B, serotype 4, subtype P1.15 sulphonamide resistant strain (B:4:P1.15). Information regarding carriage of meningococci, secretor status, ABO and Lewis blood group antigens were reported in a previous study [15]. Informed consent to participate in the survey was obtained from parents or guardians of the children.

Isolates of neisseria

An isolate of *N. lactamica* and five different isolates of *N. meningitidis* expressing different serogroup, serotype and subtype antigens were obtained from the freeze-dried strains in the collection of the Infection and Immunity Laboratory, Medical Microbiology Department, University of Edinburgh. The outbreak strain and a B:15:P1.16 sulphonamide resistant isolate were obtained from Dr R. J. Fallon, Meningococcus Reference Laboratory (Scotland), Ruchill Hospital, Glasgow (Table 1). The bacteria were grown on Modified New York City (MNYC) agar [20] for 24 h in a humidified atmosphere with 10% CO_2.

Determination of total immunoglobulin isotype levels

The total amount of IgM and IgA were assayed by a capture ELISA method. IgG from serum and saliva was coated directly onto ELISA plates. For the measurement of total IgM and IgA, the wells of polystyrene microtitre plates were coated overnight at 4 °C with either 100 μl of mouse monoclonal anti-human IgM

Table 1. *Bacterial isolates*

Strain	Source	Serogroup	Serotype	Subtype
N. meningitidis				
A11	Patient	B	15	P1.16
A43	Patient	B	4	P1.15
A41	Carrier	B	4	—
A26	Carrier	C	4	—
A48	Carrier	NG*	4	—
N. lactamica				
L01	Carrier	NG	—	—

* Non-groupable.

Clone No. MB-11, lot 69F-4807 (1/500) (Sigma, Poole, Dorset, UK) or mouse monoclonal anti-serum IgA Clone No. GA-112, lot 99F-4803 (1/500) (Sigma) diluted in coating buffer (15 mM $NaCO_3$, 35 mM NaH_2CO_3, 3 mM NaN_3; pH 9·6). The coated plates were washed three times with phosphate buffered saline (PBS) (0·16 M-NaCl, 8 mM-NaP_2HPO_4, 1 mM-KH_2PO_4, 3 mM-KCl; pH 7·2) containing 0·01 % bovine serum albumin (BSA) and Tween 20 (0·05 % v/v) (PBS-Tween) and blocked with BSA (1 %) in PBS (blocking buffer) for 30 min. After washing, 50 μl of serum (1/2000) or saliva (1/10) diluted in blocking buffer were added and incubated for 2 h at room temperature. Plates for detecting IgM were washed and 50 μl of sheep μ-chain-specific anti-human IgM (1/80) (Scottish Antibody Production Unit, Lanarkshire, Scotland) (SAPU) were added. After washing, 50 μl of horseradish peroxidase conjugated (HRP) donkey anti-sheep/goat IgG (1/20) were added to the plates for 1 h. For IgA plates, 50 μl HRP rabbit anti-human μ-chain-specific IgA (1/500) (Dako, High Wycombe, Bucks, UK) were added to the plates and incubated for 2 h.

Finally, the plates were washed and 50 μl of phosphate citrate buffer (0·1 M-$NaHPO_4$, 0·1 M citric acid) containing the substrate *O*-phenylene-diamine (0·4 mg/ml, pH 5·0) activated by 0·02 % H_2O_2 (30 % v/v). The colour was allowed to develop in the dark and the reaction was stopped after 20–30 min by adding 50 μl of H_2SO_4 (12·5 %).

To determine total IgG, 100 μl of serum (1/50000) or saliva (1/2) diluted in coating buffer were added to the microtitre plates and the plates incubated overnight under the conditions used, none of the reagents was at a limiting concentration. After washing, 50 μl of HRP sheep anti-human IgG (1/20) (SAPU) diluted in blocking buffer were added for 2 h. The plates were then treated as for IgA and IgM.

Optical density (OD) at 490 nm was determined by an ELISA plate reader (Dynatech) and corrected by subtracting the OD of the corresponding blank. Samples were tested in duplicate and the readings averaged. ELISA readings were converted to mg/ml by extrapolation from the curve that was constructed from readings obtained with standard human serum: IgG = 1140 mg/dl; IgA = 250 mg/dl; IgM = 114 mg/dl (Behring lot no. 041024). A series of twofold dilutions of standard human serum (Behring, London, UK) containing known concentrations of immunoglobulin (mg/ml) were tested with the specimens from the children.

Whole cell enzyme linked immunosorbent assay

Sera and saliva were examined for anti-meningococcal antibodies by a whole bacterium ELISA. *N. meningitidis* and *N. lactamica* were cultured overnight on MNYC agar at 37 °C. Large batches of microtitre plates were coated with bacteria to minimize variations due to different antigen preparations and coating procedures. Plates were coated overnight at 4 °C with 100 μl of one of the six bacterial isolates ($6{\cdot}6 \times 10^7$ bacteria/ml). The plates were washed and blocked with PBS containing BSA (1%). The buffer was removed and plates washed with washing buffer. Undiluted serum or saliva (50 μl) was added to the wells and incubated at room temperature for 2 h. The assay was continued in the same way as ELISA for total antibodies. The assay for total and specific antibodies were determined at the same time under the same conditions.

Statistical methods

The statistical analysis of the data was performed with the package SPSS/PC+. The results were summarized by geometric means, since the logarithmic values were more normally distributed than the raw data. The significance levels for differences between groups were examined with the Mann–Whitney U test and a P value of $< 0{\cdot}05$ was regarded as significant. The association between levels of antibodies in serum and in saliva was assessed by Spearman rank correlation.

RESULTS

Total IgA, IgG and IgM antibodies of secretors and non-secretors

The specificity of the ELISA for detection of IgM, IgG and IgA was assayed with purified IgM, IgG and IgA (data not shown). There was no cross reaction between IgA, IgG and IgM. There was no marked difference in total immunoglobulin levels between sera from secretors and non-secretors. Non-secretors had higher levels of serum IgM antibody compared with secretors, but the difference was not statistically significant. There was no difference in the levels of IgA or IgG in the saliva from secretors compared with non-secretors; but, there was significantly more total IgM in the saliva of secretors ($P = 0{\cdot}0274$) (Table 2).

There was no difference in serum or salivary immunoglobulin levels of smokers (37) compared with non-smokers (320).

Specific immune responses to Neisseria *species*

There was no marked difference in antibody levels to the isolates in sera of secretors compared with non-secretors, except for IgM to the non-groupable serotype 4 isolate (Table 2).

There were significant differences in the mean specific salivary IgM immunoglobulin levels of secretors and non-secretors. Non-secretors had significantly lower levels IgM for *N. lactamica* and each of the meningococcal isolates tested (Table 2). Statistical comparison of IgA and IgG antibody levels in saliva was also performed, but there was no difference between secretors and non-secretors. For both secretors and non-secretors, the highest levels of IgM were observed for the NG:4 isolate.

Table 2. *Geometric mean levels of total and specific IgM of secretors and non-secretors*

	Non-secretors	Secretors	*P*
Serum	(n = 107)	(n = 171	
Total IgM*	1·27	1·08	0·05
Specific			
N. lactamica	9·7	7·7	0·12
B:15:P1.16	10·5	8·5	0·15
C:4	6·0	5·3	0·25
B:4	22·4	14·7	0·11
NG:4	26·8	22·2	0·03
B:4:P1.15	5·2	4·8	0·48
Saliva	(n = 129)	(n = 208)	
Total IgM	63·0	87·0	0·027
Specific			
N. lactamica	1·8	2·5	0·0000
B:15:P1.16	2·1	3·1	0·0000
C:4	2·9	3·6	0·040
B:4	2·2	3·5	0·0000
NG:4	4·7	5·9	0·017
B:4:P1.15	1·9	2·9	0·0008

* Unit for mean total serum IgM levels are expressed in mg/ml; unit for mean total and specific salivary IgM levels and specific serum IgM levels expressed in μg/ml.

Table 3. *Geometric mean of immunoglobulin levels of carriers and non-carriers*

		Mean immunoglobulin levels (μg/ml)		
		Carriers (n)	Non-carriers (n)	*P*
Serum				
Isotype strain				
IgA	C:4	18·0 (67)	13·0 (209)	0·03
IgA	B:4:P1.15	9·5 (66)	8·4 (207)	0·02
IgM	C:4	12·4 (68)	4·1 (210)	0·0000
IgM	B:4:P1.15	8·3 (66)	6·5 (207)	0·0025
IgG*	C:4	5·3 (68)	4·2 (205)	0·01
IgG	B:4:P1.15	7·2 (64)	6·1 (207)	0·0033
Saliva				
IgA	NG:4	9·7 (89)	6·6 (243)	0·01
IgM	NG:4	6·5 (89)	5·1 (243)	0·03

* Unit for mean total serum IgG are expressed in mg/ml.

The correlation between serum and salivary antibodies

Analysis by Spearman correlation test found no correlation between the levels of IgG, IgA and IgM antibodies in serum and saliva ($P > 0·05$). This suggests that the increased levels of secretory IgM found among secretors are due to locally produced immunoglobulins.

Carriage of meningococci and immunoglobulin levels

Compared with non-carriers, carriers of meningococci had significantly higher levels of salivary IgA and IgM to a NG:4 strain and serum antibodies to the

outbreak strain and to a C:4:- isolate (Table 3); however, analysis with respect to both secretor status and carriage revealed that immunoglobulin levels still differed according to secretor status when carriage was taken into account.

There was no difference in salivary immunoglobulins to the outbreak strain; but there were significantly higher levels of IgA and IgM to the NG:4 isolate.

DISCUSSION

An association between non-secretion and meningococcal disease has been reported in studies of patients in Scotland, Iceland and Nigeria [13, 14]. In this study we tested the hypothesis that there might be differences in the humoral immune responses of secretors and non-secretors that could contribute to the apparent increased susceptibility of non-secretors to meningococcal disease. Both serum and salivary immunoglobulin levels were examined.

Conflicting results have been reported for the differences in immunoglobulin levels between secretors and non-secretors; however, most of these studies measured total amounts of immunoglobulin in serum and saliva [18, 19, 21]. Lower levels of both serum [19] and salivary IgA [18] were reported for non-secretors, suggesting that specific immune responses at the mucosal surfaces of non-secretors might be reduced compared with secretors. Blackwell and her colleagues [21] did not confirm the earlier observation with single radial immunodiffusion; higher levels of IgA were associated with the presence of meningococci in the individuals from whom the saliva was obtained. There was no difference in the mean levels of total IgA in the saliva of secretors compared with non-secretors from whom no *Neisseria* spp. were isolated.

There was no difference between secretors and non-secretors in total or specific levels of salivary IgA or IgG; however, non-secretors had significantly less total IgM in their saliva compared with secretors. These differences were also observed for salivary IgM to *N. lactamica* and to five isolates of meningococci expressing different combinations of serogroup, serotype and subtype antigens. There was no correlation between levels of serum IgM and secretory IgM in saliva, suggesting that salivary IgM is locally produced and has not leaked from the serum. Although serum and saliva of carriers had significantly higher levels of antibodies to some of the neisseria isolates, the effect of secretor status on IgM remained after adjustment for the effect of carriage.

A major biological role of high molecular weight, polyvalent IgA and IgM secretory antibodies might be to provide a first line of defence against particulate and polyvalent antigens such as bacteria. Individuals with IgM deficiency appear at risk of disseminated meningococcal disease [22, 23]. It has been demonstrated that locally produced IgM in IgA-deficient patients exhibits anti-virus activity [24].

The lower levels of secretory IgM in non-secretors might contribute to susceptibility to colonization, particularly among infants under the age of 12 months in whom secretory IgM is the major class of antibody on mucosal surfaces. The presence of secretory IgM in early infancy has been suggested to compensate for the absence of secretory IgA [7]. IgM had been detected in saliva of infants who were as young as one month of age [25]. If secretory IgM provides a crucial host

defence during this period of life the lower levels of secretory IgM found for non-secretors might contribute to their apparent susceptibility to meningococcal disease. This hypothesis is under investigation at present.

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